Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation

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Abstract **We have previously demonstrated that the brain contains about 80% of the 24S-hydroxycholesterol in the human body and that there is a net flux of this steroid from the brain into the circulation (Lütjohann, D. et al. 1996. *Proc. Natl. Acad. Sci. USA.* 93: 9799–9804). Combining previous data with new data on 12 healthy volunteers, the arteriovenous difference between levels of this oxysterol in the internal jugular vein and in a peripheral artery was found to be −10.2 ± 2.8 ng/ml (mean ± SEM) corresponding to a net flux of 24S-hydroxycholesterol from the brain of about 6.4 mg/24 h. The arteriovenous difference between levels of 24S-hydroxycholesterol in the hepatic vein and a peripheral artery of 12 other volunteers was found to be 7.4 ± 2.2 ng/ml, corresponding to a hepatic uptake of about 7.6 mg/24 h. The concentrations of 24S-hydroxycholesterol in the renal vein were about the same as those in a peripheral artery, indicating that a renal elimination is not of importance. Intravenously injected deuterium-labeled racemic 24-hydroxycholesterol was eliminated from the circulation of two human volunteers with half-lives of 10 h and 14 h, respectively. A positive correlation was found between the levels of circulating cholesterol and 24S-hydroxycholesterol. The results are consistent with a cerebral origin of most of the circulating 24S-hydroxycholesterol and suggest that the liver is the major eliminating organ. It is concluded that conversion into 24S-hydroxycholesterol is a quantitatively important mechanism for elimination of cholesterol from human brain. The possibility is discussed that circulating levels of 24S-hydroxycholesterol can be used as a marker for pathological and/or developmental changes in the brain.**—Björkhem, I., D. Lütjohann, U. Diczfalusy, L. Ståhle, G. Ahlborg, and J. Wahren. **Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation.** ***J. Lipid Res.*** **1998. 39: 1594–1600.**

Supplementary key words brain cholesterol • cytochrome P-450 • myelin • isotope dilution–mass spectrometry

In a recent study we showed that about 80% of the content of 24S-hydroxycholesterol in the human body is present in the brain (1). By measuring the levels of 24S-hydroxycholesterol in serum samples from the internal jugular vein and the brachial artery, a net flux could be demonstrated from the brain into the circulation. Based on measurements in eight subjects, the net flux of 24-hydroxycholesterol into the circulation was calculated to be about 4 mg/24 h. In view of the very low rate of cholesterol synthesis in the brain of adult primates (2), it was suggested that this flux may be of importance for cholesterol homeostasis in the brain. It was also shown that the levels of 24S-hydroxycholesterol in the circulation are markedly age-dependent with levels that were five times higher during the first decade of life than after the second decade. The levels of 24S-hydroxycholesterol in the cerebrospinal fluid, corrected for cholesterol, were higher and varied also with age in parallel with the levels in the circulation (1).

From the above information, it is evident that at least part of the 24S-hydroxycholesterol in the circulation originates from the brain. Using an $^{18}O_2$-inhalation technique it was recently shown that there is a continuous flux of newly synthesized 24S-hydroxycholesterol from rat brain into the circulation and that this flux is of the same magnitude as the rate of synthesis of cholesterol in the brain (3). The results of that study suggested that conversion of cholesterol into 24S-hydroxycholesterol may be the most important mechanism for elimination of excess cholesterol from rat brain. A prerequisite for the efficacy of this mechanism is an efficient transport of 24S-hydroxycholesterol over the blood–brain barrier. It has been shown that side-chain hydroxylated cholesterol species can be transferred in lipophilic membranes at a rate that is orders of magnitude higher than that of cholesterol itself (4).

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The microsomal fraction of brain homogenates from cows and rats is able to convert cholesterol into 24S-hydroxycholesterol (3, 5, 6). Very recently we showed that the NADPH-dependent cholesterol 24S-hydroxylase present in the microsomal fraction of rat brain has a capacity that would allow for the observed flux of 24S-hydroxycholesterol from the brain under *in vivo* conditions (3). Significant cholesterol 24S-hydroxylase activity could not be found in any other organs or tissues of rats. Next to the brain the adrenals contain the highest levels of 24S-hydroxycholesterol. It is not known whether human adrenals have a capacity to synthesize 24-hydroxycholesterol or whether this organ can contribute to the circulating levels of 24S-hydroxycholesterol. Purified sterol 27-hydroxylase from pig liver seems to have a very low capacity to synthesize 24S-hydroxycholesterol (7). At present the possibility cannot be completely excluded that the human liver can also produce small amounts of 24S-hydroxycholesterol.

If the brain is a major source of circulating 24S-hydroxycholesterol, this oxysterol could have a potential as a marker for pathological or developmental changes in the brain.

In order to evaluate cerebral production as well as hepatic and renal elimination of 24S-hydroxycholesterol, we have now measured the net fluxes of this steroid across the brain in more subjects and also determined the fluxes through the splanchnic area and the kidneys in healthy subjects. The kinetics for elimination of deuterium-labeled 24-hydroxycholesterol from the circulation has also been defined.

MATERIAL AND METHODS

Materials

Tetra-deuterium-labeled racemic 24-hydroxycholesterol used as internal standard and for the in vivo experiment (shown in Fig. 4) was synthesized as described previously (8).

Studies on healthy volunteers

Blood samples for determination of the levels of 24-hydroxycholesterol in the internal jugular vein and brachial artery were collected from 12 healthy male volunteers (aged 20–35 years) in the fasting basal state. Eight of these subjects participated in the previous investigation (1) and the results obtained from them have also been reported (1). The blood samples were taken from catheters inserted percutaneously. A thin Teflon catheter was introduced into the brachial artery and a Cournand catheter no. 7 was introduced into a peripheral vein, with the tip positioned in the internal jugular vein at the level of the orbita. Blood samples collected from the hepatic vein and the brachial artery were also obtained using the same technique from another 12 healthy male volunteers, 21–34 years of age. The samples from the hepatic vein were collected through a Cornand catheter that had been introduced percutaneously into a median antecubital vein and advanced to a right-sided hepatic vein under fluoroscopic control (9). In 6 of these subjects the hepatic plasma flow was estimated by infusion of indocyanine green at a constant rate (9). Blood samples from the renal vein and the brachial artery were obtained by the same procedure from 7 healthy male volunteers, 21–32 years of age. Four of these subjects were the same as those above.

Deuterium-labeled 24-hydroxycholesterol, 200 μg dissolved in ethanol and mixed with human serum albumin and sodium chloride solution (0.9%, w/v), was administered intravenously to a healthy volunteer, 41 years of age, weighing 95 kg. In another experiment 400 μg of the steroid was administered to another healthy volunteer, 56 years of age, weighing 93 kg. Blood samples were taken before and at specific time points after the administration (cf. Fig. 3).

Blood samples for determination of levels of cholesterol and 24S-hydroxycholesterol were also collected from 31 healthy normocholesterolemic volunteers, 14 males and 17 females, 28–57 years of age, mean 37 years.

All experiments involving human volunteers were reviewed and approved by the ethics committees at the Huddinge Hospital and the Karolinska Hospital.

Analytical methods

Levels of 24-hydroxycholesterol were assayed by isotope dilution–mass spectrometry with the use of deuterium-labeled 24-hydroxycholesterol and the same instrumentation and conditions as described previously (3, 8). The coefficient of variation of this method in the range of concentrations measured was about 4% (8).

Dilution of administered $^2H_4$-labeled racemic 24-hydroxycholesterol was determined by selected monitoring of the ions at *m/z* 413 and *m/z* 416 (M–90–43 ion in the mass spectrum of trimethylsilyl ether of unlabeled and deuterium-labeled 24-hydroxycholesterol, respectively). It should be noted that one of the four deuterium atoms in the molecule was lost in the generation of this ion. Under the conditions used with a content of trideuterium-labeled molecules ranging down to 1%, the content of deuterium could be measured with a coefficient of variation of less than 4%. All calculations were performed with use of computer-based measurements of area of the different ion tracings. In some cases cholesterol was also measured in plasma using an isotope dilution method (3).

Kinetic calculations

Standard methods for linear regression and pharmacokinetic methods were used to calculate the half-life of 24-hydroxycholesterol (10).

RESULTS

Figure 1 shows the results of previous (1) and present measurements of 24S-hydroxycholesterol in the internal jugular vein and a peripheral artery. This is an expansion of the previous study and data from eight of the 12 subjects have thus been presented previously (1). Eleven of the 12 volunteers had higher levels of the oxysterol in the internal jugular vein than in the peripheral artery. One of the subjects had the same concentration of 24S-hydroxycholesterol in the two vessels. The average arteriovenous difference between the levels of the oxysterol in the two vessels was found to be -10.2 ± 2.8 ng/ml (mean ± SEM). The results are consistent with a net flux of 24S-hydroxycholesterol from the brain into the circulation ($P = 0.004$, two-tailed paired *t*-test). This flux was estimated to be 6.4 ± 1.8 mg/24 h, assuming a constant cerebral plasma flow of 450 ml/min (11).

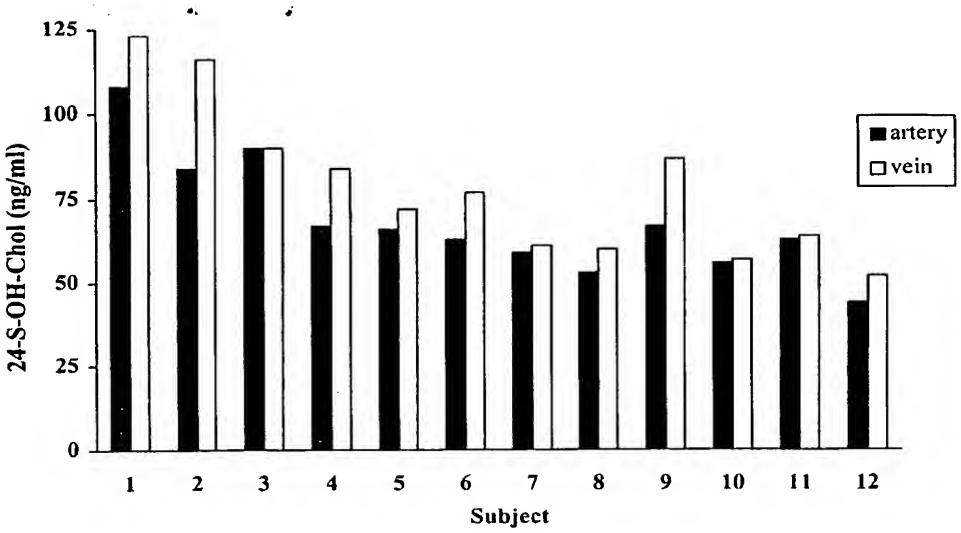

Fig. 1. Arterial-jugular venous concentration difference in levels of 24S-hydroxycholesterol. Filled bars, arterial concentrations; open bars, venous concentrations. Data from eight of the patients have been presented previously in ref. 1.

Figure 2 and Table 1 show the results of the measurements of 24S-hydroxycholesterol in the hepatic vein and in a peripheral artery. With three exceptions, there were higher levels of 24S-hydroxycholesterol in the peripheral artery than in the hepatic vein, demonstrating a net uptake of the oxysterol in the liver. In two of the three exceptions, the levels of 24S-hydroxycholesterol in the hepatic vein were almost identical to those in the peripheral artery. The arteriovenous difference was significant from a statistical point of view ($P = 0.006$, two-tailed paired t-test) and was found to be 7.4 ± 2.2 ng/ml (mean ± SEM). The hepatic plasma flow was measured in six of the subjects and found to be 0.69 ± 0.04 L/min. Using this figure for plasma flow, the net uptake of 24S-hydroxycholesterol in the splanchnic area was estimated to be 7.6 ± 2.2 mg/24 h, which is similar to, and not significantly different from, the net flux of 24S-hydroxycholesterol from the brain into the circulation.

In seven subjects the plasma level of 24S-hydroxycholesterol was measured in the renal vein and in a peripheral artery. Of the seven subjects studied, four had higher levels of the oxysterol in the renal vein and three had higher levels in the peripheral artery. The arteriovenous difference, −3 ± 4 ng/ml, was not significantly different from zero.

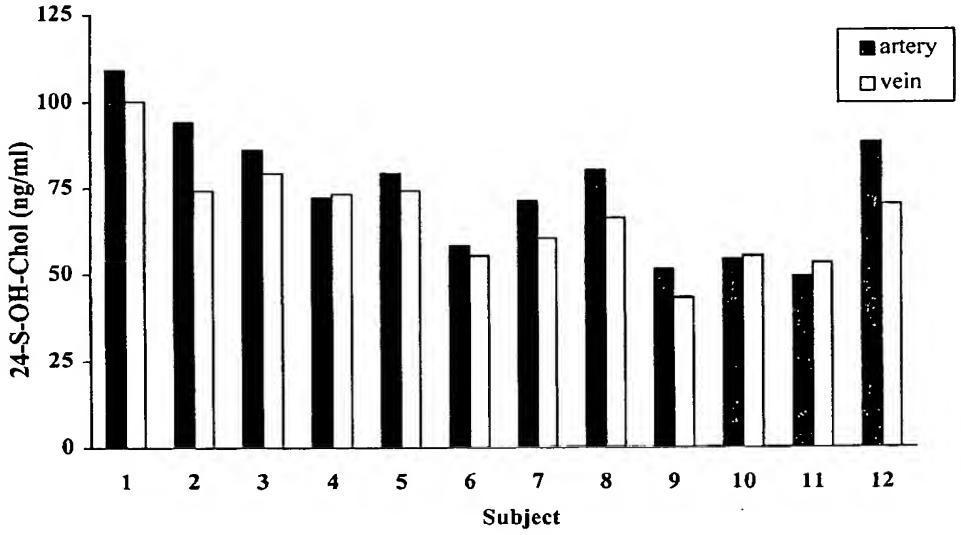

Fig. 2. Arterial hepatic venous concentration difference in levels of 24S-hydroxycholesterol. Filled bars, arterial concentrations; open bars, venous concentrations.

TABLE 1. Arterial-venous difference (A-V) and uptake of 24S-hydroxycholesterol in the splanchnic region

Subject No.	Concentration in Artery	Concentration in Vein	A-V Difference	Plasma Flow	Uptake in Splanchnic Region[a]
	ng/ml		*ng/ml*	*L/min*	*mg/24 h*
1	49	53	−4	nd	−4.0
2	51	43	+8	0.677	7.80
3	54	55	−1	0.558	−0.8
4	88	70	+18	0.767	19.9
5	79	74	+5	0.636	4.6
6	58	55	+3	0.845	3.7
7	71	60	+11	0.669	10.6
8	80	66	+14	nd	13.9
9	86	79	+7	nd	7.0
10	94	74	+20	nd	19.9
11	109	100	+9	nd	9.0
12	72	73	−1	nd	−1
Mean ± SEM	74.3 ± 5.3	66.8 ± 4.3	7.4 ± 2.2	0.692 ± 0.041	7.6 ± 2.2

[a] The uptake was calculated from the hepatic plasma flow measured and the concentration difference. In cases where the hepatic plasma flow was not measured, it was assumed to be 0.692 L/min; nd, not determined.

Using the extracerebral body content of 24S-hydroxycholesterol (4.5 mg), estimated from measurements of this oxysterol in different organs and tissues obtained at autopsy (1), and the arterial concentration of the compound (74 ng/ml), the volume of distribution could be calculated to be 60.6 L. The hepatic clearance is 4.14 L/h. From these data, the terminal half-life of intravenously administered 24-hydroxycholesterol could be expected to be 609 min (10).

In order to study the elimination of 24S-hydroxycholesterol from the circulation, deuterium-labeled 24-hydroxycholesterol was administered intravenously to a healthy volunteer. The basal plasma levels of 24S-hydroxycholesterol and cholesterol in this subject were 77 ng/ml and 1.95 mg/ml, respectively. The material injected was a racemic mixture of 24S- and 24R-hydroxycholesterol and the amount of $^2H_4$-labeled 24S-hydroxycholesterol administered containing 3 atoms of deuterium in the fragment (M-90-43) was calculated to be 200 μg. As shown in Fig. 3, there was a rapid decline in atoms percent excess deuterium during the first hour, reflecting the distribution phase with exchange with tissue 24-hydroxycholesterol. The dilution of the deuterium-labeled 24S-hydroxycholesterol followed first order kinetics between 2 and 8 h after the administration. The terminal half-life was calculated to be 603 min. Due to incomplete separation between the two isomers of 24-hydroxycholesterol, it was not possible to evaluate whether there were differences between the two stereoisomers with respect to the rate of elimination.

In another experiment with another volunteer in which the administered amount of deuterium-labeled 24-hydroxycholesterol was 400 μg, the terminal half-life was calculated to be 840 min (data not shown).

From the degree of dilution of the administered 24-hydroxycholesterol, the pool of 24S-hydroxycholesterol in equilibrium with the administered material was calculated to be about 9 mg and 10 mg, respectively. From the measured half-lives, the rate of elimination of 24-hydroxycho-

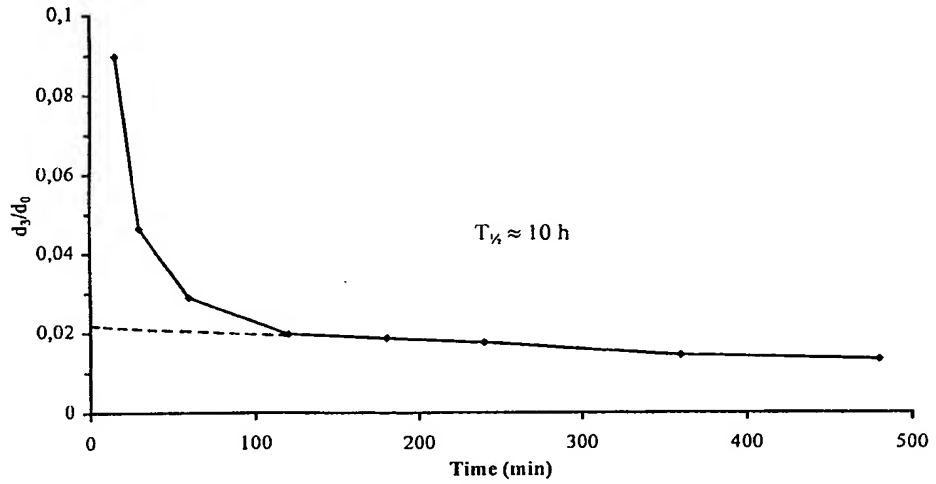

Fig. 3. Kinetics of racemic deuterated 24-hydroxycholesterol in human circulation.

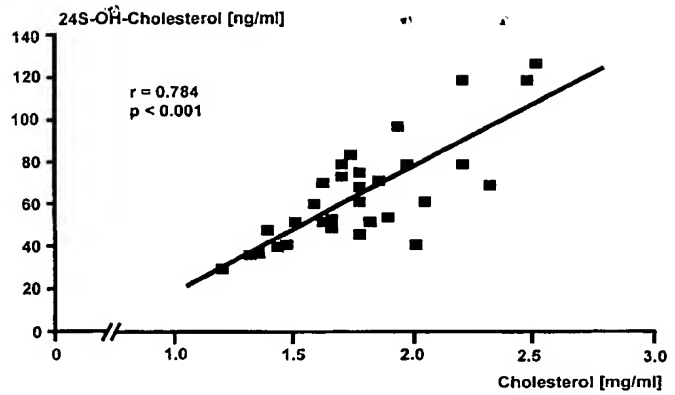

Fig. 4. Correlation between cholesterol and 24S-hydroxycholesterol in the plasma of healthy normocholesterolemic volunteers.

lesterol in the two subjects studied was estimated to be about 11 mg/24 h and 9 mg/24 h, respectively. This is of the same magnitude as the uptake of 24S-hydroxycholesterol measured in the splanchnic region (7 ± 2 mg/24 h, cf. above).

Figure 4 shows that there is a positive correlation between levels of 24S-hydroxycholesterol and cholesterol in the circulation of healthy normocholesterolemic volunteers ($r = 0.78$, $P < 0.001$). In accordance with previous work (8), no gender difference was seen.

DISCUSSION

The following results obtained here are consistent with a cerebral origin of most of the 24S-hydroxycholesterol present in human circulation: *1*) a net flux of 24S-hydroxycholesterol from the human brain into the circulation; *2*) a net uptake of 24S-hydroxycholesterol in the splanchnic region of the same magnitude as the flux from the brain; *3*) apparent absence of a renal elimination of 24S-hydroxycholesterol; and *4*) a terminal half-life of a single dose of deuterium-labeled 24-hydroxycholesterol that is consistent with data on whole-body content of 24S-hydroxycholesterol, its steady state plasma level, and the hepatic clearance.

Flux of 24S-hydroxycholesterol from the brain

The net flux of 24S-hydroxycholesterol from the brain into the circulation, based on measurements in 12 healthy volunteers, was estimated to be 6.4 ± 1.8 mg/24 h. In the previous work based on measurements in 8 of the volunteers, the net flux was calculated to be about 4 mg/24 h (1). The cerebral plasma flow was never measured and the calculation is based on the assumption that the cerebral plasma flow is about 0.45 ml/min (11).

The difference between the present result (about 6 mg/24 h) and that obtained in the previous work (about 4 mg/24 h) may be due to a combination of the analytical imprecision and the larger number of subjects used here. It should be emphasized that the mean arteriovenous difference was only about 10% and the analytical coefficient of variation was 4%. Under such conditions a relatively large variation can be predicted and measurements on relatively many subjects are necessary.

It has been calculated (12) that a transport of cholesterol from the human brain by an apolipoprotein E-dependent mechanism could account for a removal of 1–2 mg cholesterol per day. The 24S-hydroxylase-mediated mechanism thus appears to be more important than the apolipoprotein E-mediated mechanism. The two different mechanisms may together have a capacity to remove about 8 mg cholesterol from the human brain per 24 h. As the adult human brain has been reported to contain about 30 g cholesterol (13), the half-life for elimination of this cholesterol by these two mechanisms would be about 5 years. In this connection it is of interest that the half-life of brain cholesterol in adult rats has been reported to be between 2 and 6 months by different groups (3, 14, 15).

It should be pointed out that only plasma levels of 24S-hydroxycholesterol were considered here. In principle, part of the 24S-hydroxycholesterol in the circulation may be transported in erythrocytes. The concentration of 24S-hydroxycholesterol in erythrocytes was found to be about 10% of that in plasma (data not shown).

We have shown that the rate of elimination of cholesterol from the brain of rats by the 24S-hydroxylase mechanism is of the same magnitude as the rate of cholesterol synthesis in this organ (2). There is no information about the rate of synthesis of cholesterol in the human brain, but experiments in other primates (2) suggest that it is very low. Brain cholesterol is efficiently but not entirely protected from exchange with circulating lipoproteins by the blood–brain barrier (2, 14). In accordance with this, most recent studies have favored the contention that the majority of brain cholesterol is synthesized locally and not derived from the circulation (13). If the blood–brain barrier is equally effective in both directions to prevent flux of cholesterol, the importance of the present oxidative mechanism may be restricted to balance cholesterol synthesis. There is, however, also a possibility that the present oxidative mechanism compensates both for the local synthesis and for a flux of cholesterol from the circulation into the brain. The magnitude of the latter flux is not known.

Uptake of 24S-hydroxycholesterol in the splanchnic region

The average uptake of 24S-hydroxycholesterol in the splanchnic region was found to be similar or slightly higher than the average flux of 24S-hydroxycholesterol from the brain, about 7 mg/24 h. The splanchnic area includes both the intestine and the liver. It seems most likely that uptake occurs in the liver rather than in the intestine. In a previous work we measured the uptake of another side-chain hydroxylated oxysterol, 27-hydroxycholesterol, in both the intestine and the liver (16). The uptake in the liver was found to be about 5- to 6-times higher than in the intestine.

The present results are consistent with the brain as the major producer and the liver as the major eliminator of 24S-hydroxycholesterol. As there was no significant differ-

ence between the levels of 24S-hydroxycholesterol in the renal vein and in a peripheral artery, a renal elimination of 24S-hydroxycholesterol seems less likely, particularly considering that the renal blood flow is smaller than the hepatic blood flow. In accordance with this we have never found a significant excretion of 24S-hydroxycholesterol or its possible metabolites in the urine of healthy volunteers (unpublished observation). In patients with cholestasis, however, a significant such excretion of unconjugated and sulfated 24S-hydroxycholesterol seems to occur (D. Lütjohann, unpublished observation and ref. 17).

If the liver is the major eliminator of 24S-hydroxycholesterol, injected labeled 24S-hydroxycholesterol can be expected to be eliminated at a rate corresponding to the measured uptake of this compound by the liver.

Assuming a plasma flow through the liver of about 1000 L/24 h, an extracerebral pool of 24S-hydroxycholesterol of about 4.5 mg (cf. ref. 1), a plasma concentration of 24S-hydroxycholesterol of 74 ng/ml, and an uptake of 10% of this in the liver, the half-life of 24S-hydroxycholesterol in the extracerebral compartment should be about 10 h (cf. Fig. 5). In accordance with this theoretical calculation, deuterium-labeled 24-hydroxycholesterol administered to two healthy volunteers was found to be eliminated from the circulation with a $T_{1/2}$ of about 10 h and 14 h, respectively. The difference observed between the two subjects may in part be due to the analytical variation.

Figure 5 summarizes the present state of knowledge about concentrations of 24S-hydroxycholesterol in the brain and in the other tissues (1), the flux from the brain, and the uptake in the liver. In similarity with other side-chain oxidized cholesterol species (18), 24S-hydroxycholesterol may be converted into bile acids in the liver. There is, however, no information about the mechanism of this conversion. At least a small part of the 24S-hydroxycholesterol reaching the liver may be eliminated as such or as sulfate into the bile. Presence of unmetabolized or sulfated 24S-hydroxycholesterol in feces is thus well documented from previous work (19).

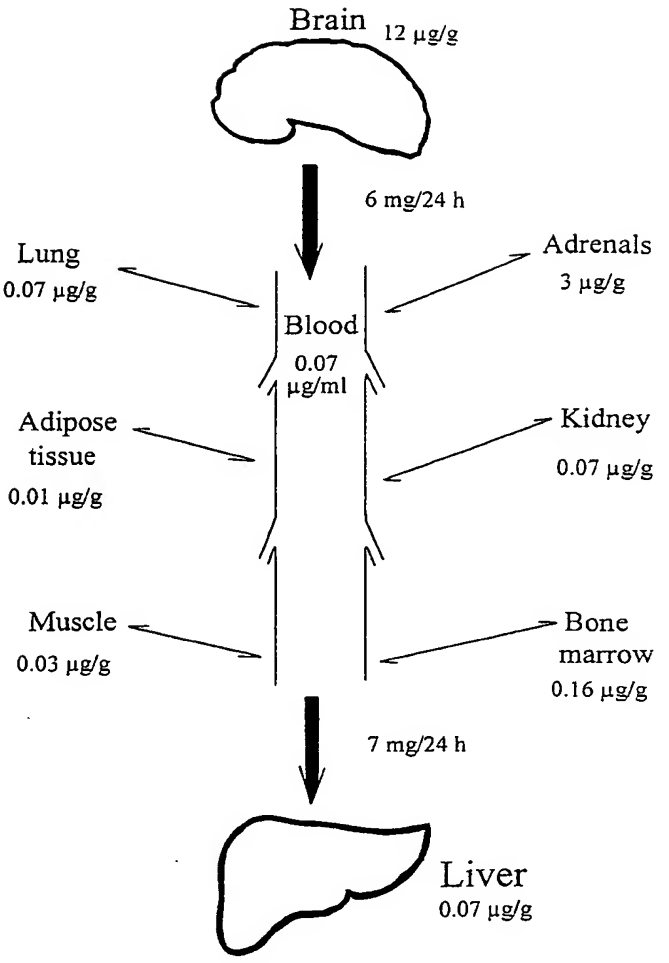

Fig. 5. Pools and fluxes of 24S-hydroxycholesterol in humans. Data on concentration of 24S-hydroxycholesterol in the different organs are from ref. 1.

Role of the cholesterol 24S-hydroxylase in the brain. Relation between levels of 24S-hydroxycholesterol and cholesterol in the circulation

Brain is not completely isolated by the blood–brain barrier (20). If the role of the microsomal 24S-hydroxylase in the human brain would be exclusively to compensate for the local synthesis, no relationship would be expected between circulating levels of cholesterol and levels of 24S-hydroxycholesterol. However, a clear positive correlation between these two levels was found here. A possible explanation is that there is some flux of cholesterol over the blood–brain barrier and that this flux is dependent upon the concentration of lipoprotein-bound cholesterol in the circulation. An important role of the cholesterol 24S-hydroxylase may then be to prevent accumulation of cholesterol in the brain caused by such a transfer, and the relationship between 24S-hydroxycholesterol and cholesterol in the circulation may be due to this. However, further work is needed to confirm this hypothesis. At the present state of knowledge the possibility cannot be excluded that the preferred substrate for the cerebral 24S-hydroxylase is cholesterol from the circulation newly transferred over the blood–brain barrier.

Can 24S-hydroxycholesterol be used as a marker for disturbances in turnover of cholesterol in the brain?

Our finding of an average uptake of 24S-hydroxycholesterol in the splanchnic region that is similar to the average flux of 24S-hydroxycholesterol from the brain, together with the absence of a renal elimination, suggests that most of the 24S-hydroxycholesterol in the circulation is derived from the brain (Fig. 5). In view of the experimental variations, a smaller contribution from other sources cannot be excluded. Adrenals contain relatively high concentrations of 24S-hydroxycholesterol (1). The total amounts of 24S-hydroxycholesterol in these organs are, however, less than 1% of the total content in the body (1), and in view of this it seems unlikely that the adrenals are important sources of circulating 24S-hydroxycholesterol. Human liver contains only very low levels of 24S-hydroxycholesterol

(1), and in view of the net uptake of 24S-hydroxycholesterol in the liver demonstrated here, it seems unlikely that a significant part of circulating 24S-hydroxycholesterol is derived from the liver.

If most of the circulating 24S-hydroxycholesterol is produced in the brain, and formation of 24S-hydroxycholesterol is an important mechanism for elimination of brain cholesterol, circulating levels of this oxysterol may reflect turnover of cholesterol in this organ. In our previous work we found a marked age-dependency in the circulating levels of 24S-hydroxycholesterol such that much higher levels are observed in young individuals than in adults. We have suggested that this may be secondary to a higher rate of cholesterol turnover in the brain in the early phases of life (1).

The above findings and considerations would make it possible to use serum 24S-hydroxycholesterol as a marker for disturbed turnover of cholesterol in the brain. The levels of 24S-hydroxycholesterol in the circulation may, however, also be dependent to some extent on the transporting capacity of the lipoproteins in the circulation and/or factors of importance for the activity of the 24S-hydroxylase in the brain. The hepatic clearance will also directly affect the plasma levels of this oxysterol. Another oxysterol in the circulation, 27-hydroxycholesterol, originates mainly from extracerebral and extrahepatic sources of cholesterol (1, 18). In a recent study we found that 24S-hydroxycholesterol and 27-hydroxycholesterol have similar distribution in circulating lipoproteins (21). In view of this, it is possible that the ratio between 24S-hydroxycholesterol and 27-hydroxycholesterol in the circulation may be a better marker for cholesterol turnover in the brain than the absolute levels of 24S-hydroxycholesterol.

Whether or not the levels of circulating 24S- and 27-hydroxycholesterol are affected in connection with various neurological disorders is now under study in our laboratory.

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ORIGINAL INVESTIGATION

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Influence of nicotine on simulator flight performance in non-smokers

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Abstract In a placebo-controlled study, we investigated the influence of nicotine on late-day aviation performance in 15 non-smoking subjects. In a within-subjects design, subjects were tested on 2 days, each lasting 8 h and consisting of three 75-min simulator flights (late-afternoon practice, evening test, night test). Prior to each test, subjects received either nicotine polacrilex 2 mg or placebo gum. As expected, overall performance was significantly better after nicotine, compared to placebo ($P < 0.01$). Post-hoc analysis of individual flight tasks showed that nicotine improved scores on approach to landing, a task which appears to require sustained attention. We conclude that nicotine may improve late-day flight performance in non-smoking aviators.

Key words Nicotine · Cognition · Psychomotor performance · Task performance and analysis · Aerospace medicine · Attention · Workload · Chewing gum · Non-smoker

Introduction

Acetylcholine systems have long been recognized to be important for cognitive functioning (Levin 1992). Nicotine, an acetylcholine receptor agonist, has been found to improve performance in smokers on tasks assessing attention, learning, reaction time and memory (Snyder et al. 1989; Sherwood et al. 1992; Warburton et al. 1992; Rusted et al. 1995; Pickworth et al. 1996). The interpretation of performance enhancements in studies conducted with nicotine-deprived smokers, however, is problematic because nicotine withdrawal leads to impaired performance, particularly on tasks requiring vigilance (Snyder et al. 1989; American Psychiatric Association 1994; Shiffman et al. 1995). Thus it can be argued that when smokers are tested following overnight smoking deprivation or shorter periods of abstinence, pre- to post-smoking improvements in performance are a result of relieving withdrawal-induced performance deficits, and therefore are not a result of nicotine per se. Indeed, nicotine withdrawal is the most consistent condition under which nicotine ingestion enhances performance. An effective way to avoid possible withdrawal deficits completely is to administer nicotine to non-smokers.

For those studies addressing the effect of nicotine on cognition in non-smokers, results have been mixed. Five studies reported significant effects of nicotine on performance (West and Jarvis 1986; Sherwood et al. 1990; Kerr et al. 1991; Le Houezec et al. 1994; Foulds et al. 1996). Four studies did not detect significant effects of nicotine in non-smokers (Wesnes and Revell 1984; Heishman et al. 1990, 1993; Hindmarch et al. 1990), including Heishman's studies which employed the same measures previously demonstrated to be sensitive to nicotine withdrawal-induced deficits, as well as their reversal by nicotine gum and patch administration in smokers (Snyder and Henningfield 1989; Snyder et al. 1989; Pickworth et al. 1996).

Two effects of nicotine that have often been found, enhanced information processing and improved sensorimotor performance (Sherwood et al. 1992), are relevant to driving a car or flying an airplane. Piloting an aircraft in particular demands a high level of psychomotor coordination, three-dimensional thinking, and alertness, and thus, is a complex information processing task comprised of many subtasks that compete for limited processing capacity. Since sophisticated measures of aviators' abilities have already been developed in our laboratory, these methods provide an

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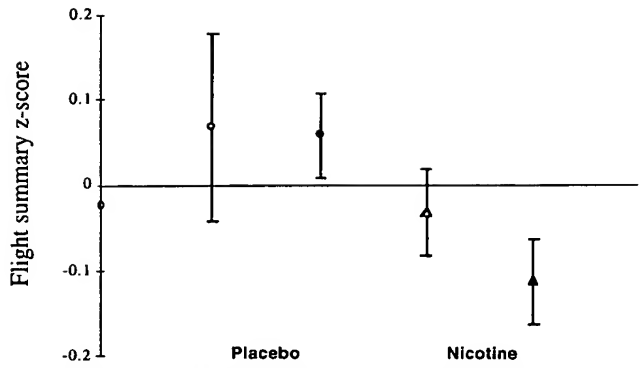

Fig. 1 Mean (± SEM) flight summary z-scores of evening and night flight on placebo and nicotine days (dose: nicotine polacrilex 2 mg prior to each evening and night flight). Lower scores mean better performance. Scores are standardized with respect to the mean and SD of the pretreatment scores. Mean practice flight performance = 0. Nicotine improved overall performance significantly, $P < 0.01$, $n = 16$. ○ Placebo evening, ● placebo night, △ nicotine evening, ▲ nicotine night

opportunity to investigate nicotine effects on complex performance. The present study examined the influence of nicotine in non-smokers, so that withdrawal relief could be ruled out as an explanation for possible post-nicotine improvements in performance. Most of the previous studies of non-smokers typically presented subjects with only one cognitive task at a time, such as finger tapping, choice reaction time, visual search, or digit recall. To our knowledge, this is the first published study that provides data about the effects of nicotine on flight simulator performance.

Materials and methods

Subjects

Subjects were seven female (mean age = 34.8, SD = 4.1) and nine male (mean age = 30.3, SD = 2.5) licensed aircraft pilots who had participated in a prior research study in our laboratory. They had an average flight experience of 1045 hours (SD = 752). All subjects were non-smokers with no history of regular smoking, and were in possession of at least a Class III Federal Aviation Administration (FAA) Medical Certificate. They were screened for health problems, consumption of nicotine and other psychoactive drugs, based on written questionnaires. Subjects were excluded if they were taking psychotropic medications or medications with arousal or sedative effects at any time during the study period. All subjects had at least 12 h of experience in the flight simulator model Frasca 141 from participation in a prior study. The protocol was approved by the Human Subject Committee of Stanford University and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All subjects gave written informed consent to participate and could withdraw at any time.

Drug conditions

All subjects received nicotine in the form of polacrilex gum, 2 mg (SmithKline Beecham Consumer Health Care, Pittsburgh, Pa., USA) or placebo gum (confectionery gum of the same size), in a within-subjects design. The gum was administered blind and to disguise the presence of nicotine, one drop of hot chili (Tabasco) sauce was added to all pieces of gum. Subjects were told they would receive either nicotine or placebo and were instructed to chew the gum slowly and steadily for 20 min.

Equipment

The equipment consisted of a Frasca 141 flight simulator (Urbana, Ill., USA) linked to a UNIX-based IRIS 3115 computer (Silicon Graphics, Mountain View, Calif., USA) that generated sophisticated "through-the-window" graphics of the environment in which the pilots flew, and collected data concerning the aircraft's flight conditions. The instrumentation, control devices (e.g., yoke, rudder pedals, throttle), and flight characteristics simulated a small, fixed landing gear, fixed propellor, single engine aircraft. The simulator used is popular as an FAA-approved pilot training device and provides realistic aircraft performance. A speaker system was installed in the cockpit and connected to a tape recorder, through which the pilot received Air Traffic Control (ATC) messages in accordance with FAA standards (FAA Order 7110.650). Each ATC script contained a take-off clearance, 16 critical enroute messages and instructions for approach and landing.

Procedures

To minimize practice effects and insure stable, relatively reliable performance, each subject participated in four training sessions prior to the first test day. In these sessions aviators performed the same tasks to be performed on the test days. On two of the four training sessions, each subject received the medication prior to the practice flight, to familiarize them with the drug. On the remaining two training sessions, no drug was administered. Pilots were tested twice a day on each of 2 test days: placebo day and nicotine day. Thus, each subject served as his or her own control. Each test day lasted a total of 8 h, starting with a pre-treatment "warm-up" practice flight at 1600 hours. After the practice flight, 30 min before starting each subsequent testing (the first test flight started at 1930 hours, the second at 2200 hours) the subjects received a single dose of either nicotine polacrilex 2 mg or placebo gum. Because of potential practice effects, the order of testing of the nicotine or placebo treatment was counterbalanced. Between the "warm-up" and the test sessions, subjects had a 1-h dinner break (1800–1900 hours).

Scenario and tasks

Each flight lasted 75 min and consisted of a standard scenario with 19 flight segments (legs) around the airport, including leg 1: take-off, leg 2–17: enroute flying, leg 18: approach, leg 19: landing. After receiving take-off clearance, pilots were given a new ATC command every 3 min with new course (heading), altitude, radio frequency, and 50% of the legs, new transponder (identification) code. In order to increase the pilots' workload, we confronted them with three different emergency situations. Carburetor icing, or drop of engine oil pressure, or suddenly approaching air traffic occurred randomly and forced the pilots to react appropriately.

Flight scoring

The scoring system of the flight simulator-computer unit produced 23 flight-performance variables. These variables were scores derived from errors or deviations from ideal or assigned positions or

values (e.g., altitude in feet, heading in degrees, airspeed in knots, reaction time in seconds). Because these individual variables had different units of measurement, it was necessary to standardize each variable to a common scale such as z-scores. We used the sample mean and SD for each individual variable at the 1600 hours pre-treatment "warm-up" flight, as the basis for the z-scores. The 23 standardized variables were aggregated into eight flight scores: take-off, communication, traffic avoidance, cockpit monitoring (consisting of oil pressure and manifold pressure scanning), approach corrections, approach course deviation, runway alignment, flare (vertical speed at touch-down). Finally, a summary score was computed, which was the mean of the flight scores of the individual variables. Summary scores may be more sensitive to drug effects because of better test-retest reliability. Prior research in our laboratory (Taylor et al. 1994, 1996) has shown high variability and little drug effect on take-off and landing scores and we have not included these two scores in the summary score. More detailed descriptions of the flight scenario and scoring are provided in Taylor et al. (1994, 1996).

Data analysis

The primary dependent measure was the flight summary score, which was computed for each treatment condition (nicotine and placebo). Secondary dependent measures were the eight individual flight scores. The summary score and the individual flight task scores were analyzed by $2 \times 2 \times 2$ mixed-model ANOVAs with Treatment (nicotine versus placebo) and Time (evening versus night) as within-subjects factors, and Order (nicotine first versus placebo first) as a between-subjects factor. All effects, main and interaction, were tested. Of primary interest, however, was the drug effect and its interaction with time. Each effect and interaction was tested as $F(1,15)$, equivalent to a two-tailed t-test with 15 degrees of freedom. Significance levels were $P < 0.05$.

Results

For the flight summary score, there was a significant main effect of Drug, [$F(1,15) = 10.7$, $P < 0.01$], with pilots performing better after administration of nicotine gum (mean = -0.07; SD = 0.40) than in the placebo condition (mean = 0.06; SD = 0.40). No significant interaction was found of Drug × Time ($F < 1$), but the means were in the direction of larger nicotine influence during the night flight. These results are illustrated in Fig. 1. No statistically significant effect was found for Time ($F < 1$), or Order ($F = 1.34$, $P > 0.2$), or any interaction (Ps > 0.2).

The individual flight task showing the largest difference between placebo and nicotine treatment was the approach to landing. In order to stay on course, pilots executed significantly more and larger corrections on the yoke during the approach [$F(1,15) = 4.9$, $P < 0.05$] in the placebo condition (mean = -0.03; SD = 0.81) compared to the flights under nicotine (mean = -0.31; SD = 0.70). A similar, but not significant drug difference was observed in approach course deviation [$F(1,15) = 3.3$, $P < 0.1$], with the trend of pilots flying the ideal approach course more accurately under the influence of nicotine. The remaining six flight tasks (take-off, communication, traffic avoidance, cockpit monitoring, runway alignment, and flare) showed no significant drug effects, or interactions (Ps > 0.05).

Discussion

In this study, nicotine polacrilex 2 mg gum given to non-smoking aviators improved overall post-treatment flight performance significantly, compared to placebo. The fact that post-nicotine improvements could be shown in non-smokers rules out withdrawal relief as an explanation in this study. It remains to be determined how much withdrawal relief contributes to the performance enhancement observed in some studies of smokers. Our investigation adds to the literature by testing the effects of nicotine on highly skilled subjects on multiple tasks of high workload and complexity set over a longer period of time, as opposed to single short-term tasks utilized in prior studies. Testing the influence of nicotine on such overall performance scores might more adequately describe the drug's impact on "real world" tasks such as flying an airplane, or operating other complex machines. However, the gained data are more difficult to compare with those from prior studies which focused on more specific, and brief tasks. The results of the present study are consistent with the findings of Le Houezec et al. (1994), who showed that subcutaneous injection of low nicotine doses in non-smokers speeded information processing on a sophisticated choice reaction time task without increasing the number of errors. The authors suggested that a positive influence of nicotine on attention may account for their findings. Our results support this account since post-hoc analyses of individual flight scores found that nicotine particularly improved performance on tasks requiring sustained attention such as the approach to landing. Generally, our study seems to confirm prior findings (Koelega 1993; Heishman et al. 1994) that vigilance tasks, requiring subjects to sustain attention, appear to be sensitive to the effects of stimulant drugs.

There may be several reasons why some studies reported statistically nonsignificant nicotine effects on cognitive performance in non-smokers following orally administered nicotine (Wesnes and Revell 1984; Heishman et al. 1990,1993; Hindmarch et al. 1990). First, these studies used tests focusing on only one cognitive task at a time, while the flight simulator test presents multiple tasks simultaneously. Nicotine-induced performance enhancement in non-smokers might not be measurable until performance tasks are complex enough to reach the limit of the subject's workload capacity. Second, only 5–12 subjects were studied in three of the four negative studies, limiting statistical power (Wesnes and Revell 1984; Heishman et al. 1990; Hindmarch et al. 1990). Third, unlike the study of Heishman et al. (1993) and most other studies, we exposed all subjects to nicotine gum repeatedly prior

to the test sessions to attenuate side-effects such as nausea, and to enhance compliance with chewing instructions, thereby increasing the possibility of detecting positive effects of nicotine on performance.

We have assessed only a single dose of nicotine, and the absence of any dose-effect data makes it more difficult to determine which effects on performance were due to nicotine itself. Future studies should address this issue. In conclusion, we found nicotine-induced aviation performance enhancement in non-smoking subjects, ruling out withdrawal-relief as an explanation for the positive effect of nicotine observed in this study. Our findings clearly show that nicotine administration not only reverses nicotine withdrawal-induced performance decrements, but also appears to improve performance on certain types of attentional tasks, confirming the same conclusions drawn in two recent reviews (Koelega 1993; Heishman et al. 1994).

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Cholesterol homeostasis in human brain: Evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation

(cerebrospinal fluid/oxysterols/plasma/stable isotopes)

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ABSTRACT **We have investigated whether side chain-hydroxylated cholesterol species are important for elimination of cholesterol from the brain. Plasma concentrations of 24-hydroxycholesterol (24-OH-Chol) in the internal jugular vein and the brachial artery in healthy volunteers were consistent with a net flux of this steroid from the brain into the circulation, corresponding to elimination of ≈4 mg cholesterol during a 24-h period in adults. Results of experiments with rats exposed to $^{18}O_2$ were also consistent with a flux of 24-OH-Chol from the brain into the circulation. No other oxysterol measured showed a similar behavior as 24-OH-Chol. These results and the finding that the concentration of 24-OH-Chol was 30- to 1500-fold higher in the brain than in any other organ except the adrenals indicate that the major part of 24-OH-Chol present in the circulation originates from the brain. Both the 24-OH-Chol present in the brain and in the circulation were the 24S-stereoisomer. In contrast to other oxysterols, levels of plasma 24-OH-Chol were found to be markedly dependent upon age. The ratio between 24-OH-Chol and cholesterol in plasma was ≈5 times higher during the first decade of life than during the sixth decade. There was a high correlation between levels of 24-OH-Chol in plasma and cerebrospinal fluid. It is suggested that the flux of 24-OH-Chol from the brain is important for cholesterol homeostasis in this organ.**

The brain is the most cholesterol-rich organ in the body. However, surprisingly little is known about the mechanism regulating cholesterol homeostasis in this organ. Very little cholesterol is taken up from circulating lipoproteins due to the efficient blood–brain barrier (1). The local synthesis of cholesterol is also very low, and it has been reported that only ≈0.1% of newly synthesized cholesterol in adult monkeys is present in the brain (2). If this is valid also in adult humans, only 1-2 mg of cholesterol would be synthesized each day. From *in vitro* experiments on slices of rat brain, it was calculated that the half-life of cholesterol is ≈6 months (3). However, the very low uptake and synthesis of cholesterol in the brain must be balanced by some mechanism for removal of cholesterol. If very little high-density lipoprotein-dependent cholesterol transport occurs, the possibility should be considered that there is a conversion of cholesterol into metabolites that may pass the blood–brain barrier more easily than cholesterol itself.

Recently, we described a new mechanism for elimination of intracellular cholesterol in macrophages, involving conversion of cholesterol into 27-hydroxycholesterol (27-OH-Chol; also denoted (25R)-cholest-5-ene-3β,26-diol) and 3β-hydroxy-5-cholestenoic acid (4). These compounds are more polar than cholesterol and easily transported out from the cells (4, 5). We have also shown that there is a continuous flux of 27-OH-Chol and other 27-oxygenated steroids from extrahepatic sources to the liver, where these compounds are rapidly metabolized into bile acids (5).

We have previously described the presence of a sterol 27-hydroxylase activity in the brain (6), and thus it appeared possible that the brain may use this enzyme to eliminate excess cholesterol.

In the present investigation we found no net flux of 27-OH-Chol from the human brain into the circulation. There was, however, a significant flux of 24-hydroxycholesterol (24-OH-Chol), and most of the 24-OH-Chol present in the circulation may originate from the brain. In addition, we show that there is a marked variation with age in plasma concentrations of 24-OH-Chol.

MATERIALS AND METHODS

Materials. Reagents and solvents used were of analytical or HPLC grade. Unlabeled and deuterium labeled oxysterols were those used in previous work from this laboratory (7).

Animals. Two male rats of an outbred Sprague–Dawley strain, 6 weeks old and weighing 130 g, were used for analysis of 24-OH-Chol in brain and serum. The animals were given free access to a standard chow and water. The experiments were approved by the animal ethics committee at the Karolinska Institute.

Human Subjects. Concentrations of 24-OH-Chol and cholesterol were determined in plasma from children and adults, 17 girls (2 months to 16 years), 19 boys (2 months to 15 years), 24 female adults (23–87 years), and 22 male adults (23–82 years). The samples were collected from healthy subjects or subjects treated for nonneurological disorders. Measurement of plasma 24-OH-Chol concentrations from the internal jugular vein and brachial artery was performed on eight healthy male volunteers (aged 20–35 years) in the fasting basal state. These blood samples were taken from catheters inserted percutaneously: one thin Teflon catheter introduced into the brachial artery and one Cournand catheter no. 7 introduced into a peripheral vein, with the tip positioned in the internal jugular vein at the level of the orbita. Diurnal variations in the levels of circulating 24-OH-Chol were determined in two healthy volunteers (31 and 39 years old). After an overnight fasting, venous blood samples were taken every 4 h during a 24-h period. Plasma samples for measurements of the degree of esterification were obtained from five healthy male adults. Measurements of 24-OH-Chol and cholesterol contents in

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Abbreviations: 24-OH-Chol, 24-hydroxycholesterol; 27-OH-Chol, 27-hydroxycholesterol; GC, gas chromatography.

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different tissues and organs were performed in autopsy materials from a 72-year-old male patient, 44 h post mortem, who died after cardiac insufficiency and chronic obstructive lung disease. Autopsy materials from brain were obtained from a man who died at the age of 80 by auricular fibrillation and cardiac insufficiency and a man who died at the age of 75 due to occipital infarction. Each sample was washed and stored in 0.1 M potassium phosphate buffer (pH 7.4), containing 2 mM EDTA and 50 μg of butylated hydroxytoluene per ml and then kept frozen at −20°C until analysis. Cerebrospinal fluid was obtained from 10 adults (24–94 years old) and 6 children (8 months–13 years) by lumbal puncture for diagnostic purposes. The adult patients suffered from various neurological disorders or were suspected to have had cerebral hemorrhage. The children were suspected to have meningitis. However, all the samples chosen for analysis were clear, colorless, and free from hemoglobin. Permission to perform all the experiments on volunteers and to collect the clinical material was obtained from the local ethical committee of Huddinge Hospital.

Plasma Oxysterols. The oxysterols were measured by isotope dilution mass spectrometry after alkaline hydrolysis, chloroform extraction, solid phase extraction, and derivatization as described (7). An individual deuterium-labeled internal standard was used for each oxysterol measured (7). Cholesterol concentrations were determined as described before (3, 8).

Degree of Esterification. The degree of esterification of plasma 24-OH-Chol with fatty acids (lipoidal esters) was determined by quantifying 24-OH-Chol before and after alkaline hydrolysis (7). An analogous procedure was performed for measurement of degree of sulfation. After addition of the internal standards and alkaline hydrolysis (stirring for 2 h with 10 ml of 0.35 M ethanolic potassium hydroxide at room temperature), the sample was acidified with 2 M sulfuric acid (pH 1) and 10 ml of ethylacetate were added before incubating for 15 h at 35°C (9). After neutralization with Na_2CO_3-solution (5% wt/vol), washing with water, and evaporating the solvent, the residue was silylated for measurement of total 24-OH-Chol by gas chromatography (GC)-MS. The results were compared with those after alkaline hydrolysis only, and the difference was regarded as the sulfated fraction.

Preparation of Samples from Different Tissues, Organs, and Different Parts of the Brain. Slices of different organs were weighed after thawing (wet weight), shortly shock-frozen again in fluid nitrogen, and freeze-dried under vacuum overnight. The dry organs were pulverized in a microdismembrator. The solid powder was weighed and the lipid components were dissolved in 6 ml of Folch solution (chloroform/methanol, 2:1) first by ultrasonicating the extract for 10 min and then stirring vigorously for 4 h at room temperature under argon gas. This solution was filtered through a usual paper filter, and the residue was washed carefully twice with 2 ml of Folch solution. Aliquots were taken for analysis. The methyl/chloroform phase contained appropriate amounts of deuterium-labeled internal standards (7). Quantitations were performed as described above for plasma oxysterols, including hydrolysis step and solid phase extraction and use of the deuterium-labeled internal standards and an appropriate standard curve (7).

24-OH-Chol in Plasma and Brain of Rats Exposed to $^{18}O_2$. The inhalation experiment was performed as described recently by Breuer and Björkhem (10). A rat was exposed to ≈20% $^{18}O_2$ (95% isotopic purity) in nitrogen in a closed system for 210 min. Plasma samples, 2 ml, were taken from a central venous catheter at 0 and 210 min from the test rat as well as from a control rat exposed to normal air under the same conditions. After collection of the final blood samples, the animals were killed by cervical dislocation. The brain was immediately isolated and treated as described above.

Analyses by GC and GC-MS. GC-analysis of cholesterol was performed on a Hewlett Packard (model HP 5890) gas chromatograph equipped with an HP1 fused silica capillary column (10 m × 0.32 mm i.d., 0.2-μm phase thickness). The oven temperature was 220°C for 1 min, then it was increased at 20°C/min to 300°C, where the temperature was kept for 6 min. Helium was used as carrier gas and flame ionization detection was performed at 290°C. 5α-Cholestane was used as internal standard. GC-MS for quantification of 24-OH-Chol, and other oxysterols in plasma and different organs was performed as described (7) using a Hewlett Packard (model HP 5890) Series II Plus gas chromatograph/HP 5972 mass selective detector. GC separation of the 24(R)- and the 24(S)-epimers of 24-OH-Chol (relation times 70.0 min and 70.8 min, respectively) was obtained on a fused silica capillary column DB 210/30 W (30 m × 0.32 mm i.d., 0.25-μm film thickness; J & W Scientific, Folsom, CA) using the same equipment as described above or on an HP 5890 gas chromatograph/HP5970 mass selective detector. The initial oven temperature was kept at 140°C for 1 min, then it was increased at 20°C/min to a final temperature of 198°C. The temperature of the transfer line was 290°C. Mass isotopomer distribution analysis was performed using the conditions described in ref. 10.

RESULTS

Content of 24-OH-Chol in Different Tissues and Organs. The absolute concentrations of nonsulfated 24-OH-Chol reached from 3.8 to 4.8 ng/mg wet weight in the cerebellum and from 8.6 to 15.1 ng/mg wet weight in the cerebrum (Table 1). Next to the brain, the adrenal glands had the highest level of 24-OH-Chol (3.4 ng/mg). In all other tissues the concentrations were <0.3 ng/mg. The cerebrum had 24-OH-Chol/cholesterol ratios between 0.68 and 2.19 ng/μg. In the other tissues this ratio reached from 0.02 ng/μg in fat tissue and 0.05 ng/μg in plasma to 0.41 ng/μg in bone marrow. When calculated from data in Table 1 and the approximate weight of the different tissues and organs, the brain was estimated to contain ≈80% of the total 24-OH-Chol content in the body. Brain samples contained also other oxysterols, but 24-OH-Chol was always dominating. The concentration of 27-OH-Chol varied between 5 and 30% of that of 24-OH-Chol whereas the concentration of 25-OH-Chol was always <3% of that of 24-OH-Chol. Since 7- and 5,6-oxygenated cholesterol species are formed in connection with autoxidation of cholesterol, it

Table 1. Absolute and cholesterol-related levels of 24-OH-Chol in tissues and organs of the human body

	24-OH-Chol, ng/mg wet weight	24-OH-Chol/Chol, ng/μg
Heart	0.11	0.35
Spleen	0.03	0.05
Liver	0.07	0.09
Adrenals	3.40	0.17
Kidney	0.07	0.10
Thymus	0.11	0.31
Duodenum	0.07	0.24
Muscles	0.03	0.26
Lung	0.07	0.21
Testes	0.04	0.11
Fat tissue	0.01	0.02
Skin	0.13	0.14
Bone marrow	0.16	0.41
Tendons	0.08	0.07
Cerebellum	3.8–4.8	0.27–0.58
Cerebrum	8.6–15.1	0.68–2.19

Cerebellum and cerebrum samples were obtained from two subjects. All the other autopsy materials were obtained from one subject only. Chol, cholesterol.

was considered to be less important to measure these steroids in the brain.

Stereochemistry at C-24 of 24-OH-Chol. 24S-hydroxycholesterol has been isolated from brain (11–14) and from adrenal glands (15). Using the chromatographic conditions described above, it was demonstrated that also plasma and liver only contain the 24S-isomer.

Degree of Esterification of 24-OH-Chol. In accordance with a previous investigation (15), 24-OH-Chol was present in the brain not only as free sterol but also as lipoidal and sulfate esters. In adrenal glands, the compound was only present as free sterol and lipoidal ester. Sulfate esters in the bovine brain have been reported to constitute ≈14% of the total content of 24-OH-Chol (15). The degree of sulfation of 24-OH-Chol in human plasma of five healthy adult volunteers was found to be 11 ± 2% (mean ± SD). In accordance with previous work (7), the mean degree of lipoidal esterification of 24-OH-Chol in human plasma was found to be ≈71%. In all subsequent work, only the sum of free and lipoidal ester fraction of 24-OH-Chol was measured.

Flux of 24-OH-Chol from the Brain into the Circulation. The levels of 24-OH-Chol in serum samples from the internal jugular vein and the brachial artery showed significant differences ($P < 0.02$, two-tailed paired t test) with higher levels in the internal jugular vein (66.3 ± 11.6 ng/ml) than in the brachial artery (58.9 ± 7.7 ng/ml). Plasma levels of 24-OH-Chol in each of the eight volunteers are shown in Fig. 1. It is evident that there is a net flux of 24-OH-Chol from the brain into the circulation. Assuming a plasma flow of 0.45 liter/min (16), this flux would correspond to ≈4 mg per 24 h.

In contrast to 24-OH-Chol, no other oxysterols among those tested (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-oxocholesterol, 25-hydroxycholesterol, 27-OH-Chol, cholestane-3β,5α,6β-triol, 5α,6α-epoxycholestan-3β-ol, and 5β,6β-epoxycholestan-3β-ol) showed a net flux from the brain.

$^{18}O_2$-Experiments for Studying Formation of 24-OH-Chol in Rats. The fact that hydroxylations catalyzed by mixed function oxidases involve introduction of one atom of oxygen from O_2 into the substrate can be utilized to measure the rate of formation of oxysterols *in vivo* with an $^{18}O_2$-inhalation technique. ^{18}O-Enrichment was measured in 24-OH-Chol isolated from brain and plasma of a rat exposed to $^{18}O_2$-containing air for 210 min (10). The fraction of 24-OH-Chol molecules containing one ^{18}O atom was 11% and 9% in brain and plasma, respectively.

Diurnal Variations in Levels of Circulating 24-OH-Chol. Levels of plasma 24-OH-Chol were measured in two volunteers at 4-h intervals over a 24-h period. No significant diurnal changes could be detected, and the levels in all six samples from both volunteers were 75 ± 6 ng/ml and 88 ± 3 ng/ml, respectively.

Age-Dependent Variations in Circulating 24-OH-Chol. There was a highly significant difference in both absolute and cholesterol-related levels of plasma 24-OH-Chol (ratio 24-OH-Chol/cholesterol) between children up to 16 years of age and adults ($P < 0.0001$) (Fig. 2). The mean levels were 157 ± 74 ng/mg cholesterol for the children and 35 ± 9 ng/mg cholesterol for the adults. The degree of sulfation of 24-OH-Chol, ≈11% of total, was the same in plasma from children as in plasma from adults.The levels remained relatively constant after the age of 20. There was no significant sex difference. The results shown in Fig. 2 were obtained both from healthy subjects (laboratory staff, medical students, and healthy volunteers) as well as from patients treated for nonneurological diseases. However, there were no significant differences between results obtained from patients and healthy subjects of the same age. It should be noted that with the exception of 27-OH-Chol, there was little or no correlation between age and levels of other circulating oxysterols. The cholesterol related levels of plasma 27-OH-Chol plotted versus age are shown in Fig. 3. The results shown in this figure were obtained from the same population as that used in Fig. 2. In this case, the levels were somewhat lower ($P < 0.001$) in the ≤20 years of age group than in the older range.

Analysis of Cerebrospinal Fluid. Cerebrospinal fluid from adults had absolute levels of 24-OH-Chol that were ≈10% of those in the circulation. No other side chain-oxidized sterols than 24-OH-Chol could be detected. The ratios between 24-OH-Chol and cholesterol were almost 10-fold higher in cerebrospinal fluid than in the circulation but less than half of those in the brain (see Table 1). In cerebrospinal fluid from 10 adults the ratio between 24-OH-Chol and cholesterol was 290 ± 50 ng/mg. In cerebrospinal fluid from six children, the corresponding ratio was more than 4-fold higher, 1690 ± 600 ng/mg. There was a very good correlation ($r^2 = 0.94$) between 24-OH-Chol/cholesterol ratios in plasma and cerebrospinal fluid (Fig. 4).

DISCUSSION

Flux of 24-OH-Chol from the Brain into the Circulation. As judged from the arteriovenous difference over the brain and the levels in cerebrospinal fluid, 24-OH-Chol was the only oxysterol among all those tested here that was transported from the human brain into the circulation. The finding in $^{18}O_2$-exposed rats that the ^{18}O-enrichment of 24-OH-Chol was similar in plasma and brain is also consistent with such a flux. It should be emphasized that the results of the $^{18}O_2$-experiment

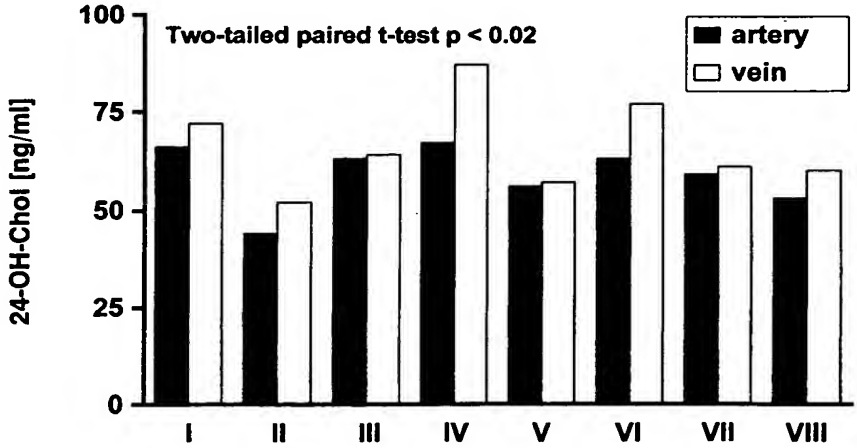

FIG. 1. Comparison of human plasma levels of 24-OH-Chol in the internal jugular vein and brachial artery in eight healthy volunteers (I–VIII).

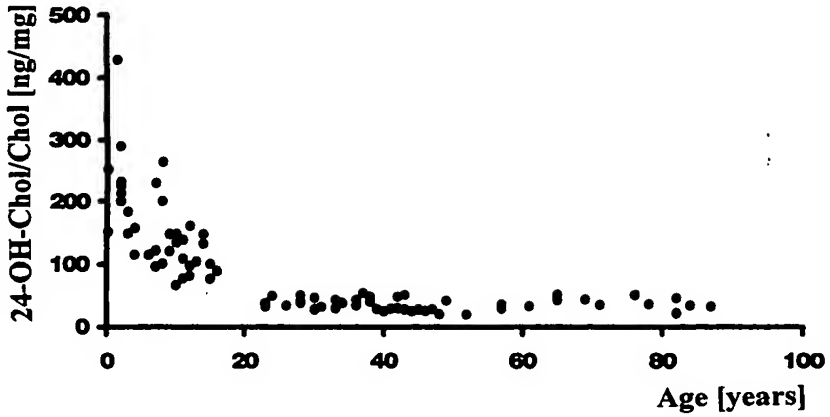

FIG. 2. Age-dependent variations in plasma 24-OH-Chol concentrations related to cholesterol (Chol). The values given are ng/mg of cholesterol.

do not exclude a bidirectional flux, in favor of the flux from the brain. The concentrations of 24-OH-Chol in the brain and adrenals were found to be similar to those reported previously (11–15, 17) and were 30- to 1500-fold higher than in any other organ. It may be mentioned that 24-OH-Chol has been denoted "cerebrosterol" in previous literature due to its occurrence in the brain (11). Heart, thymus, duodenum, muscles, and bone marrow had relatively high contents of 24-OH-Chol when related to cholesterol. The absolute levels of 24-OH-Chol in these compartments were low, however. To summarize, it seems likely that a major fraction of plasma 24-OH-Chol, being one of the predominating oxysterols in this compartment, is derived from the brain (6, 18).

It should be mentioned that the possibility of a flux of 24-OH-Chol from the brain to the circulation was suggested already 20 years ago by Lin and Smith (19).

In accordance with previous work (11), 24-OH-Chol in the brain was found to be the 24S-stereoisomer. The same stereoisomer was found in the liver, adrenals, and plasma. About 11% of the 24-OH-Chol in the circulation was sulfated, which is in good agreement with the degree of sulfation of the 24-OH-Chol reported for bovine brain (15). Since the blood–brain barrier is most effective toward polar compounds, a flux of the very polar sulfate ester of 24-OH-Chol to or from the brain seems very unlikely.

From the arteriovenous difference (brachial artery and internal jugular vein) in levels of unsulfated 24-OH-Chol, the magnitude of the total flux from the brain of adults was estimated to be ≈4 mg per 24 h. In relation to the reported very low turnover of cholesterol in the brain, such a flux is likely to be of importance for cholesterol homeostasis in this organ. This could be regarded as a selective mechanism for conversion of cholesterol into a more polar product that is transported from the brain and further converted into bile acids at a much higher rate than cholesterol itself. From the concentration of 24-OH-Chol in the cerebrospinal fluid and the flux of cerebrospinal fluid into the internal jugular vein (≈0.5 liter per 24 h), it can be calculated that <1% of the total flux of 24-OH-Chol from the brain can occur via cerebrospinal fluid. Thus, 99% of the flux of 24-OH-Chol must occur through the blood–brain barrier. Side chain-hydroxylated oxysterols are known to be transferred through lipophilic membranes orders of magnitude faster than cholesterol (20), and thus it seems likely that there is a preference for 24-OH-Chol in the elimination of sterols through the blood–brain barrier. If there is no discrimination in the transfer at all between cholesterol and 24-OH-Chol, the flux of cholesterol through this barrier would be about 1000-fold higher than that of 24-OH-Chol. Such a high flux, corresponding to a turnover of ≈25% of brain cholesterol within 24 h, is incompatible with previous demonstrations of an extremely low turnover of cholesterol in the brain (1, 2). On the other hand, a very small lipoprotein-mediated exchange of cholesterol over the blood–brain barrier is likely to occur. Such an exchange has been demonstrated in some *in vitro* models (21, 22), but not *in vivo*.

Elimination of Circulating 24-OH-Chol. If there is a continuous flux of 24-OH-Chol from the brain, the final elimination of this compound must occur in the liver or by the kidneys. In the liver, side-chain hydroxylated C_{27}-steroids are efficiently converted into bile acids (23). In an investigation by Ramsey

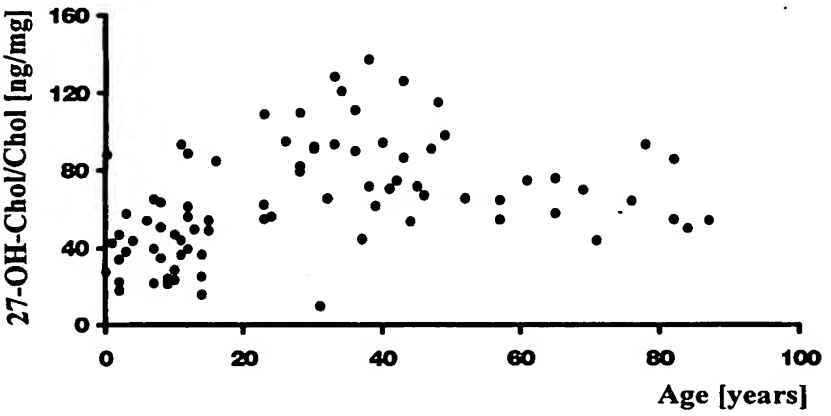

FIG. 3. Age-dependent variations in plasma 27-OH-Chol concentrations related to cholesterol (Chol). The values given are ng/mg of cholesterol. The results shown are obtained from the same population as in Fig. 2.

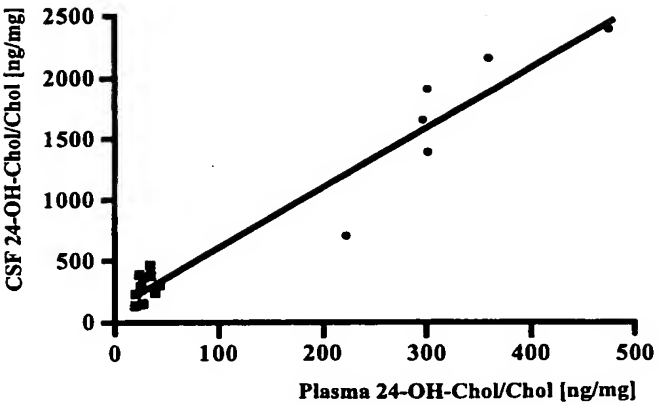

FIG. 4. Relation between levels of 24-OH-Chol in plasma and cerebrospinal fluid (CSF) related to cholesterol. Plasma samples were taken from children and adults.

and Nichols (24), labeled cholesterol was injected intracerebrally in rats. The urine of these rats was shown to contain small amounts of two oxysterols covalently bound to short-chain peptides. The two oxysterols were tentatively identified as 24- and 27-OH-Chol. Attempts in our laboratory to find covalently bound 24-OH-Chol in urine of healthy volunteers have failed hitherto, however. In this connection, it may be mentioned that 24-OH-Chol fatty acid esters appear to accumulate in the human aorta with advancing age and severity of aortal atheroma (25).

Origin of Circulating 24-OH-Chol and Capacity of the 24-Hydroxylating System in the Brain. 24-Hydroxylase activity toward cholesterol has been reported to occur only in liver mitochondria (26, 27) and in brain microsomes (14). However, the enzymes involved have never been characterized in detail. It seems likely that species of cytochrome P450 are involved and, in accordance with this, both brain microsomal and liver mitochondrial 24-hydroxylase activity toward cholesterol are dependent on NADPH as cofactor.

In a recent work from this laboratory, we showed that a cytochrome P450 containing sterol 27-hydroxylase purified to apparent homogeneity from pig liver mitochondria has a low but significant capacity to catalyze 24-hydroxylation of cholesterol (28). It is thus likely that sterol 27-hydroxylase is responsible for the low cholesterol 24-hydroxylase activity reported to be present in liver mitochondria. Patients with the rare inborn disease cerebrotendinous xanthomatosis are lacking or have a dysfunctional sterol 27-hydroxylase in the liver and other organs (29). Since such patients have normal levels of 24-OH-Chol in the circulation (unpublished data) it is evident that mitochondrial sterol 27-hydroxylase cannot be responsible for the formation of the 24-OH-Chol present in the circulation. In view of the relatively high level of 24-OH-Chol in the brain, and the flux of 24-OH-Chol from this organ, a putative brain microsomal 24-hydroxylase is likely to be responsible for a major portion of the 24-OH-Chol in the circulation. Contributions from other tissues and organs containing 24-OH-Chol (see Table 1) cannot be excluded, however.

The 24-hydroxylase present in brain microsomes has a very low activity. Furthermore, the assay in a crude preparation of brain microsomes is complicated by the dilution of added substrate by high amounts of endogenous cholesterol. Dhar *et al.* (14) reported a maximal conversion of 0.4% of added labeled cholesterol into 24-OH-Chol after 24 h of incubation. Preliminary experiments in our laboratory with microsomes, whole homogenates, and slices of brains from rats and mice have resulted in similar or lower degrees of conversion, and attempts to characterize the enzyme in detail have failed thus far. Evidence has been presented that the brain contains species of cytochrome P450 capable of 27-, 7α-, and 11β-hydroxylation of steroids (6, 30, 31) and steroid side-chain cleavage (31, 32). Very recently Stapleton *et al.* (33) described a novel cytochrome P450 (hct-1) that is expressed primarily in the brain (33). The natural substrate for this enzyme was not defined, but it was believed to be of steroid nature. At present the possibility has to be considered that hct-1 is the species of cytochrome P450 responsible for 24-hydroxylation of cholesterol.

Information about the quantitative importance of the enzymatic mechanism for removal of brain cholesterol in rats can be obtained from the present $^{18}O_2$-experiments. Under the conditions employed, ≈11% of the total pool of 24-OH-Chol in the brain of the rat was replaced with newly synthesized material during 210 min of exposure. Since the overall ratio between 24-OH-Chol and cholesterol in the brain of a rat is ≈1:250, the finding is consistent with a conversion of ≈0.01% of all brain cholesterol into 24-OH-Chol per hour. Since the equilibration of the enzyme system with $^{18}O_2$ may have been 60% rather than 100% under the conditions employed (10), the rate of conversion may have been even higher. This degree of conversion is similar to that demonstrated by Dhar *et al.* (14) *in vitro*. Theoretically, the half-life for elimination of all cholesterol present in a rat brain by this mechanism would then range between 6 and 10 months. This elimination may be compared with the estimated half-life of cholesterol in slices of rat brain of ≈6 months (3). Most probably the half-life of cholesterol is considerably longer in the human brain.

Age-Dependent Variation in Levels of Circulating 24-OH-Chol. The finding that the levels of plasma 24-OH-Chol in infants are markedly higher as compared with adults could be due to a higher flux of this steroid from the brain. The higher levels of 24-OH-Chol also in the cerebrospinal fluid from infants and the correlation between these levels and those in the circulation give strong support for this contention. The possibility that there is a lower rate of peripheral metabolism of 24-OH-Chol in younger than in older subjects seems unlikely in view of the fact that age had little or no effect on other similar oxysterols (25- and 27-OH-Chol) that may be metabolized by the same enzymes. It should be mentioned that in a study by Smith *et al.* (17), there was no clear effect of age on the concentration of 24-OH-Chol in human brain. However, in that study, no brain samples were analyzed from subjects younger than 16 years old. In a previous study on side-chain hydroxylated cholesterol species in the steroid sulfate fraction of feces, Gustafsson and Sjövall (34) found that the ratio between 24-OH-Chol and cholesterol was higher in fractions from younger than from older infants. This is in accordance with the findings in the present study.

Overall synthesis of cholesterol in the body is markedly higher in young subjects than in old. It has been reported that throughout the life of a rat, from rapidly developing fetus to adult, there is a 10-fold decrease in the rate of whole animal synthesis of cholesterol (1). To our knowledge, there is no specific information about the influence of age on cholesterol synthesis in the human brain. If 24-hydroxylation is of importance for cholesterol homeostasis, an increased flux of 24-OH-Chol would compensate for part of a higher synthesis of cholesterol at early age. On the other hand, 24-OH-Chol is known to be a potent inhibitor of cholesterol synthesis (35). Thus it is possible that both removal of cholesterol and inhibition of cholesterol biosynthesis may be under regulation of the 24S-hydroxylase. Further work is needed to establish the mechanism behind the age-dependent variations in the levels of circulating 24-OH-Chol and its possible relation to cholesterol synthesis.

Can 24-OH-Chol Be Used as a Marker for Pathological and/or Developmental Changes in the Brain? Organ-specific compounds are used extensively as diagnostic tools, and there

are circulating markers for most of the major organs in the body. The brain is, however, an exception. As long as the blood–brain barrier is intact, there is a very low flux of low molecular weight compounds from the brain into the circulation. The possibility that 24-OH-Chol may be used for neurodiagnostic purposes is being investigated at present in our laboratory.

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Plasma levels of 24S-hydroxycholesterol reflect the balance between cerebral production and hepatic metabolism and are inversely related to body surface

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Abstract **We have previously presented evidence that most of the 24S-hydroxycholesterol present in the circulation originates from the brain and that most of the elimination of this oxysterol occurs in the liver. Plasma 24S-hydroxycholesterol levels decline by a factor of about 5 during the first decades of life. The concentration of the enzyme cholesterol 24S-hydroxylase in the brain is, however, about constant from the first year of life, and reduced enzyme levels thus cannot explain the decreasing plasma levels during infancy. In the present work we tested the hypothesis that the plasma levels of 24S-hydroxycholesterol may reflect the size of the brain relative to the capacity of the liver to eliminate the substance. It is shown here that the age-dependent changes in absolute as well as cholesterol-related plasma level of 24S-hydroxycholesterol closely follow the changes in the ratio between estimated brain weight and estimated liver volume. The size of the brain is increased only about 50% whereas the size of the liver is increased by about 6-fold after the age of 1 year. Liver volume is known to be highly correlated to body surface, and in accordance with this the absolute as well as the cholesterol-related plasma level of 24S-hydroxycholesterol was found to be highly inversely correlated to body surface in 77 healthy subjects of varying ages ($r^2 = 0.74$). Two chondrodystrophic dwarves with normal size of the brain but with markedly reduced body area had increased levels of 24S-hydroxycholesterol when related to age but normal levels when related to body surface. It is concluded that the balance between cerebral production and hepatic metabolism is a critical determinant for plasma levels of 24S-hydroxycholesterol at different ages and that endocrinological factors are less important. The results are discussed in relation to the possibility to use 24S-hydroxycholesterol in the circulation as a marker for cholesterol homeostasis in the brain.**—Bretillon, L., D. Lütjohann, L. Ståhle, T. Widhe, L. Bindl, G. Eggertsen, U. Diczfalusy, and I. Björkhem. **Plasma levels of 24S-hydroxycholesterol reflect the balance between cerebral production and hepatic metabolism and are inversely related to body surface.** *J. Lipid Res.* **2000.** 41: **840–845.**

Supplementary key words liver volume • cholesterol 24S-hydroxylase • brain size • brain cholesterol homeostasis

24S-hydroxycholesterol is one of the major oxysterols in human circulation. We have shown that most of this compound originates from the brain, and that there is a continuous flux of it from the brain into the circulation (1, 2). This flux is likely to be an important part of the cholesterol turnover in the brain, and the flux of 24S-hydroxycholesterol from a rat brain seems to be similar to the rate of synthesis of cholesterol in that organ (3). As judged from the arteriovenous concentration difference, the liver seems to be the major eliminator of circulating 24S-hydroxycholesterol (2).

There is a strong age-dependent variation in the levels of circulating 24S-hydroxycholesterol (1). Newborns have very low circulating levels of 24S-hydroxycholesterol (D. Lütjohann, I. Björkhem, S. Locatelli, C. Dame, J. Schmolling, K. von Bergmann, and H. Fahnenstich, unpublished observation) but these levels increase rapidly after birth and are highest at an age of about 1–2 years (1). The levels decline by a factor of about 5 during the first two decades of life and then remain about constant.

24S-hydroxycholesterol has a high affinity for the LXRα receptor (4) and the possibility has been discussed that this oxysterol is an important part of a signalling system from the brain (5). In addition it is evident that plasma levels of 24S-hydroxycholesterol may reflect turnover of cholesterol in the brain and may be used as a marker for disturbances in this turnover. Very recently we showed that a specific population of patients with Alzheimer disease and vascular dementia had slightly but significantly higher levels of 24S-hydroxycholesterol than controls (6). In view of these findings, it was considered to be important to define the factors affecting plasma levels of 24S-hydroxycholesterol and, in particular, to explain the mechanism behind the dramatic decrease in these levels during infancy and puberty. This decrease may be due to reduced

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enzymatic activity, reduced levels of the enzyme cholesterol 24S-hydroxylase in the brain, reduced levels of substrate (free cholesterol) for the enzyme, or increased metabolism of the oxysterol in the liver.

In a very recent work, antibodies towards cholesterol 24S-hydroxylase were prepared and used for immunoassay of the enzyme in human brain (7). The levels of the enzyme in the brain were found to be about constant from the age of 1.5 years and onwards. Decreasing enzyme levels are thus not the explanation for the decreasing plasma levels of the oxysterol during infancy.

In the present work results are presented suggesting the balance between cerebral production and hepatic metabolism to be the most important determinant for plasma levels of 24S-hydroxycholesterol. The age-dependent changes in the plasma levels of the oxysterol were found to closely follow the age-dependent changes in the ratio between estimated brain weight and liver volume. This was found to be valid also in chondrodystrophic dwarves with normal size of the brain but with reduced body size. In consonance with this, the circulating levels of 24S-hydroxycholesterol were found to be inversely correlated to body surface.

MATERIALS AND METHODS

Subjects

A total of 205 adults of both sexes (120 women, 85 men, aged 21 to 86 years from the Swedish population) are included in the study presented in Table 1 and Fig. 1. All these adults regard themselves as healthy and had no medications. The samples were taken in the morning in the fasting state.

Some of the adults (n = 29) presented in Table 1 were defined with respect to body height and weight and are included in the study shown in Figs. 4 and 5. In addition, three healthy adults with exceptional body height were recruited for the study presented in these figures. Table 1, Figs. 4 and 5 also included data from 48 infants and children of both sexes from 1 to 18 years of age. The plasma samples had been collected for diagnostic purposes and ethical permission was obtained to use the excess of these plasma samples for the present study. In most cases, the infants could only be defined with respect to sex and age. All these infants were treated at the hospital due to a medical or surgical illness. Samples from a few apparently healthy well-defined infants who were hetero- or homozygotes with respect to sitosterolemia were also included (n = 3 and 2, respectively), as well as 9 well-defined infants with short-bowel syndrome, Ullrich-Turner syndrome, atrium-septum defect, chronic diarrhea, or Mb Crohn. The levels of 24S-hydroxycholesterol from the latter infants were similar to those of infants of the same age with an undefined medical or surgical illness. The weight and height of the infants and children could only be defined in the latter two groups (n = 14). The brain weight and the liver volume of the above 78 subjects (30 adults and 48 infants and children) were calculated using literature data (8–10).

Two chondrodystrophic dwarves were included in the study. The male dwarf was 48 years of age, had a length of 150 cm and a weight of 70 kg. He had been operated in the back due to spinal stenosis a few days before collection of the blood sample, but was otherwise healthy. The female dwarf was 54 years of age, had a length of 123 cm and a weight of 52 kg. She was bound to a wheel-chair due to spinal stenosis at the time of the collection of the blood sample, but was otherwise healthy.

The study was done in accordance with the principles of the Declaration of Helsinki, and ethical permission for collection of plasma samples was obtained from the Ethical Committee of Huddinge Hospital.

Analyses and analytical methods

Serum concentrations of cholesterol were measured by standard enzymatic procedures (CHOP-method, Boehringer Inc., Mannheim, Germany). Levels of 24S-hydroxycholesterol were assayed by isotope dilution mass spectrometry using racemic [23, 23, 24-$^2H_3$]deuterium-labeled 24-hydroxycholesterol and the instrumentation and conditions previously described (1–3, 6, 11). The intra- and interassay coefficient of variation of this method is about 4% and 8%, respectively.

Turnover models and statistics

Previous data suggest that the production of 24S-hydroxycholesterol is of cerebral origin while the elimination is hepatic (2). Assuming that the production is proportional to the brain size, the elimination is proportional to the liver size, and assuming that the volume of distribution does not affect the plasma concentration, due to the steady-state conditions, we get:

$$dC_p/dt = k_p \cdot BrW - k_e \cdot C_p \cdot LW = 0$$
$$\text{thus, } C_p = k_p \cdot BrW / k_e \cdot LW$$

where C_p is the plasma concentration of 24S-hydroxycholesterol, BrW is the brain weight, LW is the liver weight, and k_p and k_e are proportionality constants for formation and elimination, respectively. Literature data were used to estimate the brain weight (8) and the liver volume (9, 10). In the latter case, volume was substituted for weight assuming a density of 1.0.

Ordinary linear regression was used to analyze the relation between 24S-hydroxycholesterol and BrW/LW.

RESULTS

In **Table 1** results of measurements of plasma levels of 24S-hydroxycholesterol and cholesterol are presented from 48 infants and 205 adults of both sexes, covering an age interval from 1 to 86 years. The adults are all subjectively healthy. Most of the infants have some type of non-neurological disease, and the samples had been collected for routine diagnostic purposes. For ethical reasons most of the infants could only be defined with respect to age and sex.

Figure 1 shows the relation between plasma levels of 24S-hydroxycholesterol and cholesterol in the whole material of adults subjects (n = 205). In accordance with a previous study with fewer subjects (2), there was a high correlation between the two parameters with a r^2 value of 0.44. In view of the high correlation, the levels of 24S-hydroxycholesterol are presented below both as absolute concentration (ng/mL) and as the ratio between the level of 24S-hydroxycholesterol and that of cholesterol (ng/mg).

The results presented in Table 1 confirm the previously reported decline in both absolute and cholesterol-related plasma levels of 24S-hydroxycholesterol during infancy and puberty. A noteworthy finding was that the levels during decades 6 and 7 were slightly but significantly higher than the levels during decades 3–5. There was no consis-

TABLE 1. Absolute and cholesterol-related plasma levels of 24S-hydroxycholesterol in 253 human subjects (48 infants and 205 adults of both sexes)

Age Group	Age	n			24S-Hydroxycholesterol	24S-Hydroxycholesterol/Cholesterol
		Total	Female	Male		
	yr				*ng/mL*	*ng/mg*
1	1-5	12[d]	6	2	385 ± 64	391 ± 73
2	6-9	11[d]	2	8	258 ± 31	240 ± 29
3	10-18	25[d]	14	10	192 ± 12[a]	157 ± 9[b]
4	19-30	37	20	17	77 ± 3[c]	65 ± 2[c]
5	31-40	30	13	17	76 ± 4	59 ± 2[a]
6	41-50	47	28	19	77 ± 4	56 ± 2
7	51-60	49	36	13	87 ± 3[a]	58 ± 2
8	61-70	20	11	9	105 ± 7[b]	67 ± 2[b]
9	71-86	22	12	10	101 ± 5	64± 3

Results are expressed as means ± SEM.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; significantly different from the corresponding value of the previous age.

[d] The reason for the differences observed between the total number of subjects and the number of females plus males is that some infants can only be defined with respect to age.

tent significant sex difference in any of the different age groups.

In the previous work (2) we showed that the brain is the major producer and that the liver is the major eliminator of 24S-hydroxycholesterol. Given the fact that the concentration of the enzyme is about constant after the age of 1.5 years (7), the ratio between the size of the brain and the size of the liver is likely to be of importance for the levels of 24S-hydroxycholesterol in the circulation.

Figure 2 shows the age-dependent changes in the brain weight and liver volume reported from autopsy studies (8), and studies with computed tomography scanning (9) or ultrasound technique (10). The ratio between brain weight and liver volume decreases with a factor of 4 during infancy and puberty, followed by a slight increase during the later decades of life. It should be mentioned that there are some sex-related differences in brain weight and liver volume, whereas the ratio between the two parameters is affected very little by sex (in general the difference is less than 10%).

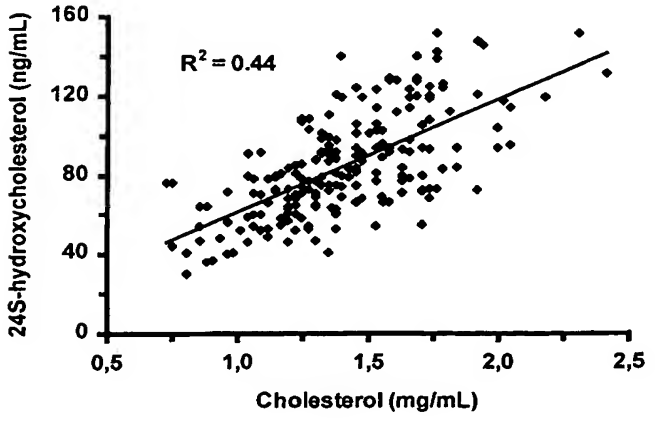

Fig. 1. Relation between plasma levels of 24S-hydroxycholesterol and cholesterol in adult subjects (from 21 to 86 years old, 120 women, 85 men).

Figure 3 shows the levels of absolute (A) and cholesterol-related (B) levels of 24S-hydroxycholesterol from the age of 1 year and onwards. The ratio between brain weight and liver volume for the different ages, based on literature data (8–10), is also indicated in the figure. The levels of 24S-hydroxycholesterol closely follow the ratio between brain weight and liver volume during infancy and puberty as well as during later decades of life.

If the ratio between the brain and the liver is the major determinant for plasma levels of 24S-hydroxycholesterol, endocrinological factors during puberty should be less important. Adult chondrodystrophic dwarves have a normal head size but a markedly reduced body size. Except for reduced levels of receptors for growth hormone in the skeleton, they are normal from an endocrinological point of view. As indicated in Fig. 3B, two chondrodystrophic dwarves of opposite sexes had highly significant increased cholesterol-related levels of 24S-hydroxycholesterol (101 ng of 24S-hydroxycholesterol per milligram of cholesterol for both of them). This finding supports the contention that the high levels of 24S-hydroxycholesterol during infancy and puberty are due to the relation between the size of the brain and the size of the body (including the liver) rather than due to endocrinological factors.

A very high correlation between liver size and body surface area during the first three decades of life has been reported (9). An inverse correlation between levels of 24S-hydroxycholesterol and body surface area would therefore be expected. The absolute (results not shown) as well as the cholesterol-related plasma levels of 24S-hydroxycholesterol (**Fig. 4**) have been plotted against body surface area in 77 subjects of different ages from 1 to 86 years. There was a high inverse correlation in both cases ($r^2 = 0.65$ and 0.74, respectively). The plasma cholesterol-related levels of 24S-hydroxycholesterol and the estimated body surface area of the two dwarves have also been indicated.

Body surface area is well correlated to body height (12, 13). Thus an inverse correlation between levels of 24S-hydroxycholesterol and body length would also be expected. The correlation (r^2) between absolute levels of

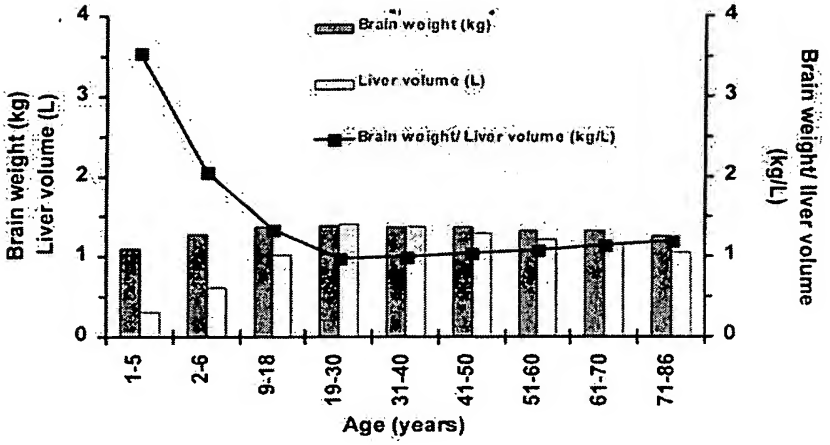

Fig. 2. Age-related changes in brain weight (adapted from ref. 8) and liver volume (adapted from refs. 9 and 10).

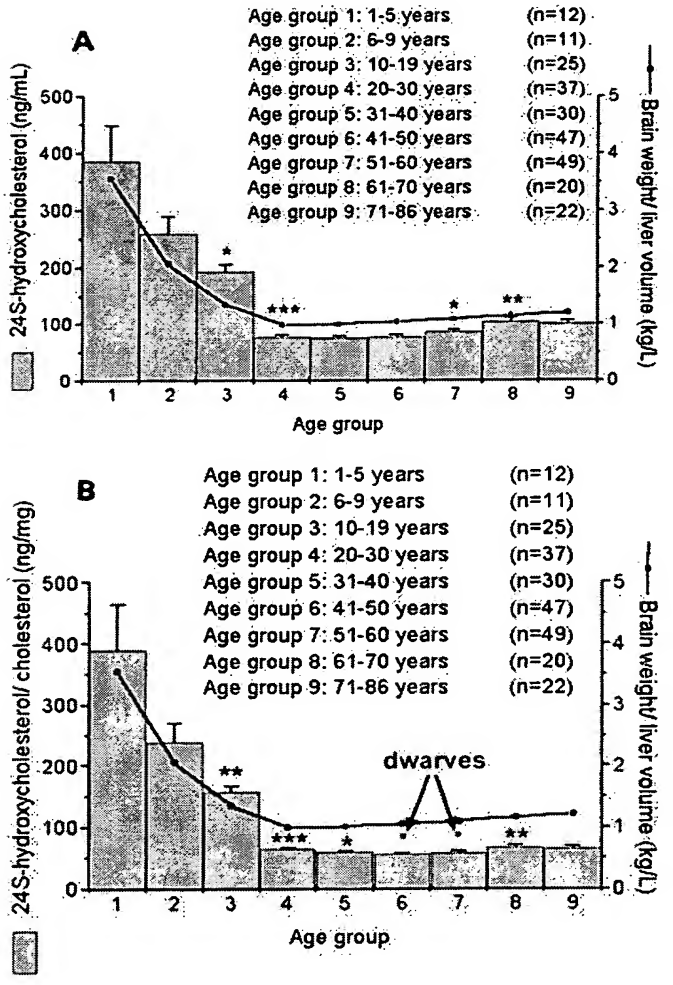

Fig. 3. A: Absolute and B: cholesterol-related levels of 24S-hydroxycholesterol in human subjects at different ages (48 infants and 205 adults). Results are expressed as means ± SEM. Estimated ratio between brain weight and liver volume is indicated (cf. Materials and Methods); *, **, and ***: significantly different from the corresponding value of the previous age at $P < 0.05$, 0.01, and 0.001, respectively.

24S-hydroxycholesterol and body length was found to be 0.62 and the corresponding correlation for cholesterol-related levels of 24S-hydroxycholesterol and body length was found to be 0.71 (results not shown). The corresponding correlations to body weight were found to be 0.64 and 0.73, respectively (results not shown).

DISCUSSION

The background to this study was our previous result that there are arteriovenous concentration differences over the liver and the brain, indicating that the brain produces and the liver eliminates 24S-hydroxycholesterol (2). This model predicts that the plasma level of 24S-hydroxycholesterol should vary with the ratio between brain

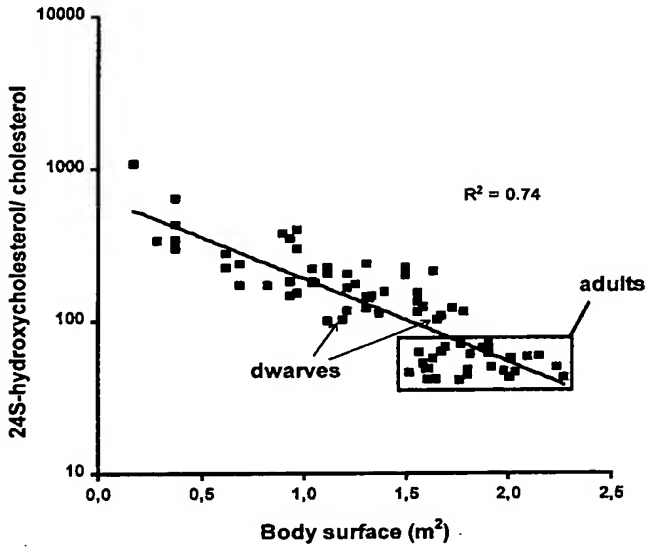

Fig. 4. Relation between plasma cholesterol-related levels of 24S-hydroxycholesterol and body surface in 77 subjects (48 infants and 29 adults of both sexes). Values of cholesterol-related levels of 24S-hydroxycholesterol have been plotted in a logarithmic scale.

weight and liver weight, as shown above. The results obtained in this study are in accordance with the prediction and support our hypothesis.

The high correlation between circulating levels of 24S-hydroxycholesterol and cholesterol observed in the present work is in accordance with previous observations in smaller populations (2, 6). The possibility has been discussed that part of the explanation for this may be that there is a small flux of cholesterol from the circulation over the blood–brain barrier into the brain (2). The distribution of 24S-hydroxycholesterol in the lipoproteins is, however, similar to that of cholesterol (14) and changes in the cholesterol pool are thus likely to lead to changes in the distribution volume for the oxysterol and consequently also in the concentration of this oxysterol. The plasma concentration of the structurally similar oxysterol 27-hydroxycholesterol has also been found to be highly correlated to the plasma level of cholesterol (15). A consequence of the high correlation between plasma levels of 24S-hydroxycholesterol and cholesterol is that the ratio between 24S-hydroxycholesterol and cholesterol is likely to be a better marker for the cerebral production than the absolute concentration of the oxysterol.

The evident age-dependent changes in the circulating levels of 24S-hydroxycholesterol seem to be unique, as all other oxysterols measured in the circulation vary little with age (1). It should be pointed out that production of 24S-hydroxycholesterol occurs in an organ that increases little in size after the age of 2 years (about 30%) whereas elimination of 24S-hydroxycholesterol involves an organ that increases in size up to 6-fold during this period.

If the capacity of the liver to metabolize 24S-hydroxycholesterol is proportional to the size of the organ, this is a likely explanation for the age-dependent variations. The blood flow through the liver must be of importance for the clearance rate, and it is known that there is a good correlation between hepatic blood flow and the size of the liver (10). During the last decades of life, the liver size decreases more than the brain size (cf. Fig. 2). If the metabolism of 24S-hydroxycholesterol is proportional to liver size, slightly increased levels of 24S-hydroxycholesterol would be expected during the last decades of life. That such an increase could be demonstrated lends further support for the contention that the balance between cerebral production and hepatic metabolism is most important for the circulating levels of this oxysterol.

The enzymes involved in hepatic metabolism of 24S-hydroxycholesterol have not been defined in detail. The relatively long half-life of 24S-hydroxycholesterol in the circulation, 10–14 h (2), suggests that the enzymes involved are less effective than those involved in the metabolism of other oxysterols. We have shown that the cholesterol 7α-hydroxylase (CYP7A) present in human liver has at least some activity towards 24S-hydroxycholesterol (M. Norlin, A. Toll, I. Björkhem, and K. Wikvall, unpublished observation), but the quantitative importance of this specific cytochrome is not known. Oxysterol 7α-hydroxylase (CYP7B) does not seem to have any activity towards 24S-hydroxycholesterol (M. Norlin, A. Toll, I. Björkhem and K. Wikvall, unpublished observation). It is noteworthy that treatment of patients with ketoconazol, which is an inhibitor of CYP7A and also other species of cytochrome P-450, causes an increase in the levels of 24S-hydroxycholesterol (I. Björkhem and U. Diczfalusy, unpublished observation). It is well documented that cholestasis causes increased levels of 24S-hydroxycholesterol in the circulation (16, 17), and it is evident that the metabolic capacity of the liver is a critical factor for the circulating levels of 24S-hydroxycholesterol.

There was a high inverse correlation between body surface area and plasma levels of 24S-hydroxycholesterol (r^2 = 0.74, Fig. 4). Because there is a high correlation between body surface area and liver volume (10), such a correlation would be expected. In this connection it is interesting that chondrodystrophic dwarves, with normal size of the brain but with markedly reduced body size, had high cholesterol-related levels of 24S-hydroxycholesterol in the circulation. This finding supports the contention that the ratio between size of the brain and size of the liver is the most important determinant for circulating levels of 24S-hydroxycholesterol.

In previous works (1, 2), we discussed the possibility that the high levels of 24S-hydroxycholesterol in the circulation of infants and children may be secondary to a high turnover of brain cholesterol during this period of life. The results presented here suggest that most of the age-dependent variation in levels of 24S-hydroxycholesterol reflects a balance between a relatively constant production of the compound in the brain and an age-dependent elimination of the compound in the liver (Fig. 5). To our knowledge, there are few other examples of this. The continuous relatively constant production of creatinine in the muscles and the elimination of this compound by the kidneys has some similarity with the mechanism studied here.

Plasma levels of 24S-hydroxycholesterol are not subjected to diurnal variations (1) and with the exception of changes due to liver disease (16, 17), neurological disorders (6), and drugs, the levels seem to be relatively stable

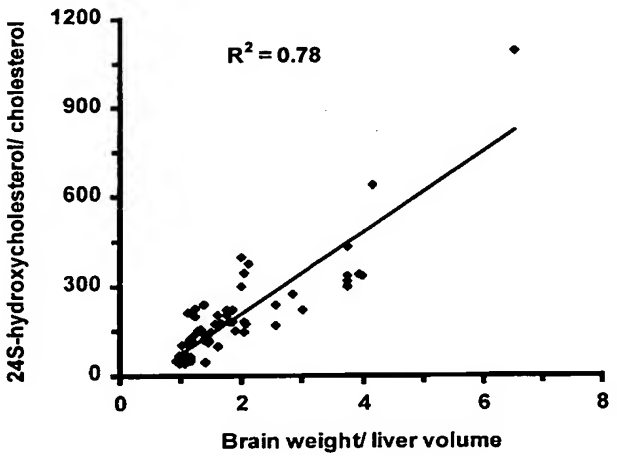

Fig. 5. Relation between plasma cholesterol-related levels of 24S-hydroxycholesterol and estimated brain weight to liver volume ratio in 77 healthy subjects.

in adults. In view of this it seems less likely that 24S-hydroxycholesterol is of major regulatory importance in extracerebral tissues. It is evident, however, that plasma levels of cholesterol and body surface area should be taken into account when evaluating circulating levels of 24S-hydroxycholesterol in different populations.

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Randomized Trial of the Effects of Simvastatin on Cognitive Functioning in Hypercholesterolemic Adults

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PURPOSE: In our initial study of the potential effects of cholesterol-lowering interventions on cognitive functioning, treatment with lovastatin as compared with placebo caused performance decrements on several neuropsychological tests, whereas scores on other tests were unaffected. The current study was designed to confirm and extend those findings.

METHODS: The study comprised 308 hypercholesterolemic adults between 35 and 70 years of age. Employing a randomized double-blind design, we assigned participants to daily treatment with placebo, 10 mg of simvastatin, or 40 mg of simvastatin for 6 months. A neuropsychological test battery was administered to assess cognitive functioning at baseline and at the end of the treatment period.

RESULTS: A total of 283 subjects completed the study: 94 subjects on placebo, 96 taking 10 mg of simvastatin, and 93 taking 40 mg of simvastatin. Compared with placebo, decremental effects of simvastatin treatment were found on tests previously observed to be sensitive to statins ($P = 0.008$; difference in summary z scores = 0.18; 95% confidence interval [CI]: 0.07 to 0.29) and on tests not previously administered ($P = 0.04$; difference in summary z scores = 0.17; 95% CI: 0.05 to 0.29), but not on tests previously observed to be insensitive to statins ($P = 0.84$; difference in summary z scores = 0.02; 95% CI: -0.07 to 0.10). For the three tests specifically affected by simvastatin, effects on cognitive performance were small, manifest only as failure to improve during the 6 months of treatment (compared with placebo), and were confounded by baseline differences on one test.

CONCLUSION: This study provides partial support for minor decrements in cognitive functioning with statins. Whether such effects have any long-term sequelae or occur with other cholesterol-lowering interventions is not known. **Am J Med. 2004; 117:823–829.** ©2004 by Elsevier Inc.

High serum cholesterol level is a widely recognized risk factor for coronary artery disease, the leading cause of death in western industrialized countries. For most patients attempting to lower their serum cholesterol levels, the primary medical intervention is daily, long-term use of a statin.

Initial studies of the adverse effects of statins have been reassuring, although these agents can cause liver injury, myopathy, and peripheral neuropathy (1,2). There also has been recent interest in their potential effects on brain function. It has been suggested that statin therapy reduces the risk of Alzheimer disease (3), but recent clinical trials have found no treatment effect on dementia (4,5). In fact, one review of 60 cases of statin-associated memory loss, along with other reports of depression, sleep disorders, and global amnesia, raise questions about possible adverse effects on the brain (6).

In 2000, we reported the results of our initial study of central nervous system effects of statins (7). The investigation employed a double-blind, randomized, placebo-controlled design to evaluate the effects of lovastatin on cognitive functioning and mood among 209 middle-aged adults with hypercholesterolemia. Compared with masked placebo, 20 mg of lovastatin taken daily for 6 months had detrimental effects on cognitive performance on four neuropsychological tests assessing attention, working memory, and overall mental efficiency. Performance on other cognitive tests and mood were not affected substantially. The observed treatment effects were quantitatively small and were primarily manifest not as an absolute decline in performance but as a failure to improve upon repeat post-treatment testing. Learning or practice effects occur commonly upon readministration of many neuropsychological tests, even though equivalent, alternative versions of the tests are frequently employed to minimize such learning effects. The current trial was undertaken to confirm and extend the observations made in our initial investigation.

METHODS

Subjects were generally healthy men and women between 35 and 70 years of age with mild-to-moderate hypercho-

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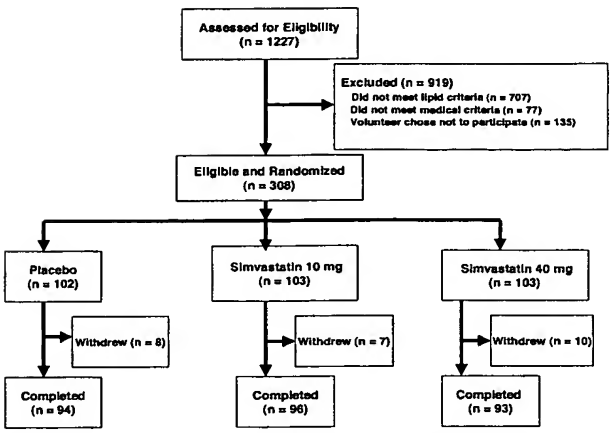

Figure. Flow diagram of progression of subjects through the phases of the study.

lesterolemia, defined as a low-density lipoprotein (LDL) cholesterol level between 160 and 220 mg/dL. Participants were recruited from Allegheny County in southwestern Pennsylvania by mass mailings of the study brochure and placement of media advertisements. The study protocol was approved by the University of Pittsburgh Institutional Review Board, and informed consent was obtained from all subjects.

Exclusion criteria included the following medical conditions: secondary hyperlipidemia (lipid disorders attributable to chronic hepatitis, renal failure, or untreated hypothyroidism), severe hypertriglyceridemia (fasting serum triglyceride level >350 mg/dL), coronary artery disease, stroke, diabetes, untreated hypertension (diastolic blood pressure >95 mm Hg), cancer, and major neuropsychiatric conditions (e.g., schizophrenia, seizures, dementia). Subjects were also excluded if they reported current treatment with any lipid-lowering medication or supplement, psychotropic medication, glucocorticoid, or opiate analgesic. Sexually active premenopausal women were excluded unless they were using birth control.

Sample size calculations indicated that 100 subjects per condition (n = 300 total) would provide at least 80% power to detect an effect size of $f = 0.25$ between placebo and 10 mg of simvastatin, and between 10 and 40 mg of simvastatin from baseline to post-treatment (i.e., group by time interaction) when using a two-tailed F-test from a repeated-measures analysis of variance with a significance level of 0.01. The effect size measure (f) is the standard deviation of the standardized means; from a behavioral science perspective, an effect size of 0.25 would translate into a medium-sized effect (8).

A total of 1227 subjects attended at least one screening visit, of whom 443 (36%) met all eligibility criteria (Figure). Of these 443 volunteers, 308 (70%) completed the screening procedures. Following stratification by race (black vs. other), age (≤50 vs. >50 years), and sex, each participant was assigned randomly to daily treatment with placebo, 10 mg of simvastatin, or 40 mg of simvastatin. Permuted block randomization within strata for each combination of the three factors was used to generate the treatment assignment lists. All three treatments were encapsulated and identical in appearance to preserve blinding of subjects and research staff. The treatment period was 6 months.

Two hundred and eighty-three of the randomized participants (92%) completed the investigation. The number of withdrawals did not differ by treatment condition, although the reasons varied. Withdrawals due to suspected adverse treatment reactions included 4 among subjects receiving 40 mg of simvastatin and 3 among those receiving 10 mg of simvastatin; none in the placebo group had adverse reactions. Withdrawal due to a serious adverse event occurred in only 1 subject who suffered a stroke while taking 40 mg of simvastatin. Compliance was monitored by pill counts at interim follow-up appointments (weeks 8 and 16) and at the final visit (month 6). Compliance averaged 95% over the treatment period and did not differ by condition.

Measurements

Fasting blood samples for serum lipid determinations were drawn twice during screening (separated by 1 to 3 weeks), after 2 and 4 months, and twice during the last month of treatment. The two baseline lipid measurements were averaged, and the two post-treatment measurements were averaged. Determinations of serum total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were performed by the Heinz Nutrition Laboratory, Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, which has met the criteria of the Centers for Disease Control and Prevention–National Heart, Lung, and Blood Institute Lipid Standardization Program since 1982. LDL cholesterol level was calculated using the Friedewald equation (9).

In our initial investigation (7), a broad neuropsychological assessment battery was administered, and four individual tests showed statistically significant effects of statin treatment: Digit Vigilance, Recurrent Words, Elithorn Mazes, and Grooved Pegboard. In the current trial, we assembled a neuropsychological test battery containing statin-sensitive tests, comprising the four tests revealing drug effects in our initial investigation; statin-insensitive tests, including six tests from our initial investigation that had not detected drug effects, readministered to verify the specificity of statin effects; and new tests, including the Mirror Tracer and 4-Word Short-Term Memory tests, chosen because of their sensitivity to small differences in cognitive performance among high-functioning persons (Appendix).

Table 1. Baseline Characteristics of Subjects by Treatment Group*

Characteristic	Placebo (n = 94)	Simvastatin 10 mg (n = 96)	Simvastatin 40 mg (n = 93)
	Number (%) or Mean ± SD		
Age (years)	54.1 ± 8.7	53.0 ± 9.9	54.2 ± 8.7
Female sex	50 (53)	51 (53)	46 (50)
White	84 (89)	82 (85)	78 (84)
Weight (kg)	81.7 ± 13.6	82.8 ± 13.2	84.0 ± 15.4
Blood pressure (mm Hg)	125/80 ± 12/8	125/81 ± 14/9	125/82 ± 13/8
Education (years)	14.8 ± 3.2	15.0 ± 3.7	14.7 ± 3.3
Alcohol (drinks per week)	2.4 ± 1.2	2.6 ± 2.2	2.3 ± 1.3
Total cholesterol (mg/dL)	261 ± 20	261 ± 21	266 ± 22
LDL cholesterol (mg/dL)	180 ± 16	180 ± 15	183 ± 15
HDL cholesterol (mg/dL)	51 ± 11	50 ± 12	53 ± 18
Triglycerides (mg/dL)	150 ± 69	152 ± 62	152 ± 60
Vocabulary (NAART errors)	24 ± 10	24 ± 13	24 ± 12
Fluid intelligence (Block Design scaled score)	8.7 ± 2.2	8.7 ± 2.9	8.7 ± 2.6

* The treatment groups did not differ on any listed characteristic.
HDL = high-density lipoprotein; LDL = low-density lipoprotein; NAART = North American Adult Reading Test.

Testing sessions were conducted at baseline and at the end of treatment. Alternative forms of the tests, where available, were used in randomized order at the baseline and post-treatment assessments. Prior to baseline testing, subjects attended a practice session to familiarize themselves with test materials and instructions. To examine treatment effects on health-related quality of life, participants completed the Medical Outcomes Study Short-Form General Health Survey (21).

Statistical Analysis

In several instances, continuous variables were subjected to transformation to ensure normality of distribution. Baseline characteristics were compared using analysis of variance, the Kruskal-Wallis test, or the chi-squared test, as appropriate. The effects of treatment on serum lipid concentrations and quality of life were examined with repeated-measures analysis of variance.

To limit experiment-wise error, the neuropsychological data were grouped into the following categories: statin-sensitive tests, statin-insensitive tests, and new tests. Repeated-measures multivariate analysis of variance was utilized to analyze the multiple test scores in each category simultaneously. Treatment type and test form were between-subjects factors and visit (baseline and post-treatment) was the within-subjects factor. The two levels of active treatment (10 and 40 mg of simvastatin) were collapsed in the initial analyses, and then separated in subsequent analyses to test for a dose-response relation. Effects of drug treatment were indicated in the omnibus F statistic for the interaction of treatment type with visit. Significant treatment effects observed in multivariate analyses were followed by univariate analyses to identify on which neuropsychological test or tests performance differed by treatment assignment. All analyses utilized two-tailed P values, with $P \leq 0.05$ indicating statistical significance.

In a second parallel set of analyses conducted to estimate effect sizes, we computed standardized z scores (individual test score minus mean test score divided by the standard deviation) at baseline and post-treatment using the mean and standard deviation of the baseline tests. Where necessary, scores were multiplied by -1 so that higher scores always indicated better performance. Summary scores for the three test categories were constructed by averaging the z scores of the constituent tests. Change in cognitive performance was calculated by subtracting the summary z scores at baseline from the summary z scores post-treatment. A t test for each of the test categories was conducted to examine the effects of treatment assignment on change in cognitive performance. These analytic procedures were those employed in our initial investigation (7) and are in accord with multivariate statistical analyses used in other studies examining change in cognitive performance based on multiple neuropsychological tests (22). Analyses were performed using SPSS, version 11 (Chicago, Illinois).

RESULTS

The subjects were generally middle-aged with moderate hypercholesterolemia (Table 1). Most had attended some college, and scores on tests of vocabulary and problem-solving abilities generally were above average. Comparison of the treatment groups with respect to the 13 demographic and clinical variables and the 12 neuropsychological test scores at baseline indicated that the groups were similar except on the Recurrent Words test (placebo vs. treatment groups: 79.6% vs. 84.0%, $P = 0.04$).

Table 2. Summary Z Scores* of Cognitive Function at Baseline and after 6 Months of Treatment

Neuropsychological Test Category	Placebo Group (n = 94)		Simvastatin Group (n = 189)		Group Difference in Change	*P* Value
	Baseline	Post-Treatment	Baseline	Post-Treatment		
	Mean ± SD				Mean (95% Confidence Interval)	
Statin-sensitive tests	−0.05 ± 0.61	0.15 ± 0.56	0.02 ± 0.61	0.04 ± 0.59	0.18 (0.07 to 0.29)	0.002
Statin-insensitive tests	0.00 ± 0.60	0.09 ± 0.54	0.00 ± 0.63	0.08 ± 0.65	0.02 (−0.07 to 0.10)	0.72
New tests	−0.07 ± 0.81	0.19 ± 0.84	0.03 ± 0.76	0.13 ± 0.80	0.17 (0.05 to 0.29)	0.007

* Higher scores indicate better performance.

Median treatment adherence based on pill counts during the 6 months of treatment was 95% and did not differ among the treatment groups. Serum lipid concentrations changed little in the placebo-treated subjects during the treatment period. Subjects assigned to 10 mg of simvastatin experienced an average decline in total cholesterol level of 54 mg/dL (95% confidence interval [CI]: 49 to 60 mg/dL), about 21% as compared with baseline, whereas the total cholesterol level of participants receiving 40 mg of simvastatin fell by 80 mg/dL (95% CI: 75 to 85 mg/dL), or about 31%.

In multivariate repeated-measures analysis of variance of the statin-sensitive neuropsychological tests, simvastatin altered cognitive performance compared with placebo ($P = 0.008$). A similar result was noted when the data were analyzed using the difference in change in summary *z* scores (score = 0.18; 95% CI: 0.07 to 0.29; $P = 0.002$). Examination of the *z* scores indicated that on statin-sensitive tests subjects receiving placebo improved between baseline and post-treatment visits, whereas those assigned to simvastatin did not (Table 2).

In analyses of the statin-insensitive tests, performance scores were unaffected by treatment (multivariate $P = 0.84$; summary *z* score = 0.02; 95% CI: -0.07 to 0.10). There was an effect of treatment on the new neuropsychological tests, based on either multivariate analysis ($P = 0.04$) or *t* test of summary *z* scores (score = 0.17; 95% CI: 0.05 to 0.29; $P = 0.007$). Cognitive performance on the new tests improved more from baseline to post-treatment in subjects taking placebo than in those receiving simvastatin (Table 2).

In analyses of the mean scores on the individual neuropsychological tests comprising the statin-sensitive and new categories (Table 3), statistically significant treatment effects were observed on scores on Elithorn Mazes ($P = 0.02$) and Recurrent Words ($P = 0.04$) tests in the statin-sensitive category, whereas performance on the Grooved Pegboard ($P = 0.09$) and Digit Vigilance ($P = 0.84$) tests was not significantly affected. Performance improved on the Recurrent Words and Elithorn Mazes tests in placebo-treated subjects but not in those receiving simvastatin. The groups, however, differed at baseline on the Recurrent Words test. On the two new tests, decremental effects of simvastatin reached statistical significance on the 4-Word Memory test ($P = 0.05$) but not on the Mirror tracing test ($P = 0.09$). Again, subjects receiv-

Table 3. Performance on Individual Tests of Cognitive Function at Baseline and after 6 Months of Treatment

Test*	Placebo Group (n = 94)		Simvastatin Group (n = 189)	
	Baseline	Post-Treatment	Baseline	Post-Treatment
	Mean (95% Confidence Interval)			
Statin-sensitive tests				
Digit Vigilance (errors)†	6.2 (4.7–7.6)	5.7 (4.4–7.0)	6.9 (6.0–7.9)	6.2 (5.1–7.3)
Recurrent Words (% correct)	80 (76–83)	83 (80–86)	84 (82–86)	85 (83–86)
Elithorn Mazes (seconds)†	172 (160–184)	144 (133–155)	162 (154–170)	160 (152–168)
Grooved Pegboard (seconds)†	144 (139–149)	144 (138–150)	147 (142–152)	150 (145–154)
New tests				
Mirror Tracing (errors)†	48 (40–56)	40 (32–48)	43 (38–48)	41 (35–46)
4-Word Memory (no. correct)	18.9 (17.4–20.4)	20.8 (19.1–22.4)	19.4 (18.3–20.4)	20.0 (18.9–21.1)

* Descriptions of individual tests are provided in the Appendix.
† Higher scores indicate worse performance.

ing placebo improved in performance whereas those receiving simvastatin did not.

When the two active treatment groups (10 mg and 40 mg) were compared to test for the presence of a dose-response relation, we found that the 40-mg dose of simvastatin did not have greater effects on cognitive performance than the 10-mg dose ($P > 0.15$). In addition, neither overall quality of life nor the mental component scale scores differed in placebo and simvastatin-treated participants ($P > 0.15$).

DISCUSSION

In our re-examination of the effects of statins on cognitive functioning, we found that treatment adversely affected performance on neuropsychological tests that were sensitive to lovastatin in our initial investigation. Performance on new tests was also negatively affected by simvastatin, as compared with placebo. However, these effects were rather circumscribed; statistically significant treatment effects were observed on just three of six tests when analyzed individually, and baseline differences confound interpretation of one of these measures. The cognitive decrements with the 40-mg dose of simvastatin were no greater than those observed with the 10-mg dose, suggesting a threshold effect. As in our initial investigation, the treatment effects were small and were manifest not as an absolute decline in performance but as a lack of improvement between baseline and post-treatment assessments. Indeed, learning or practice effects are observed upon readministration of most neuropsychological tests within 1 year (22,23) and can obscure small or modest drug effects.

Cognitive function is often divided into domains or skill areas, such as memory, psychomotor speed, attention, and mental flexibility. In our studies, the neuropsychological tests on which performance was affected by statins tap a variety of skills, and therefore our findings indicate that such cognitive effects are not specific to one or two domains of function. Tests found to be sensitive to the effects of statins tended to have relatively large learning or practice effects, as evidenced by the improvement seen in placebo-treated participants. This suggests that the effects of statins on cognition may affect patients' abilities to benefit from prior experience or devise performance-enhancing strategies.

Even though the brain contains five to 10 times as much cholesterol as other organs, and most of its dry weight is lipid (24,25), few studies have examined the effects of variation in serum cholesterol level or cholesterol reduction on the brain. Familial hypobetalipoproteinemia, a genetic disorder resulting in hypocholesterolemia, is associated with spinocerebellar degeneration and dementia (26). Inhibition of hydroxymethylglutaryl coenzyme A reductase with statins impairs learning processes in rodents (27,28), and large doses of statins have neurotoxic effects in dogs (29,30). In cross-sectional studies, low (untreated) serum cholesterol concentration has been associated with relatively poor performance on some cognitive tests (31,32). Conversely, age-related cognitive decline has been associated with either low (33,34) or high (35,36) serum cholesterol level.

Few randomized clinical trials have been reported. Wardle and colleagues assigned 176 hypercholesterolemic volunteers to either of two cholesterol-lowering diets or a waiting-list control condition (37). Scores on three cognitive tests were unaffected by treatment, but performance on a test measuring sustained attention was poorer in both diet groups compared with controls, and performance decrement correlated with the decline in serum cholesterol level. A small industry-sponsored study of statin therapy found deleterious effects of lovastatin on tests measuring vigilance and divided attention, whereas two other similar studies reported no effects on cognitive performance (38–40). Cognitive assessments were unaffected in the elderly in two placebo-controlled trials of statin treatment conducted for other purposes (4,41). However, one study administered only one neuropsychological test and the other study administered only three tests; neither study used measures that we have found to be sensitive to statin treatment. As noted earlier, observational studies have suggested that statin use may decrease the risk of Alzheimer disease (3), but recent clinical trials have not reported any treatment effects on the incidence of dementia (4,5).

The mechanism by which statins may affect cognitive function is not known, but given the broad effects of these drugs on cellular metabolism, there are several possibilities (42,43). For example, statins might alter neuronal function through effects on brain cholesterol metabolism (44–46), ubiquinone, protein prenylation (47), vitamin E (48–50), or omega-3 fatty acids (51,52). We recently observed that simvastatin increases the preponderance of omega-6 over omega-3 fatty acids (53).

The results of this investigation stand as a partial replication of our earlier trial. Given the limitations of the findings, the evidence of decremental effects of statins on cognitive functioning remains preliminary. Further study is warranted because of both the extremely widespread use of these drugs and the decline in cognitive function that accompanies aging. Treatment effects may differ by patient group or with nonstatin cholesterol-lowering interventions, and may either amplify or resolve (via development of tolerance) with long-term treatment. In any case, this research challenges the current orthodoxy that cholesterol is of importance only as a risk factor for atherosclerosis.

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Appendix. Neuropsychological Test Battery

Statin-Insensitive Tests	
Elithorn Mazes*	Planning and drawing time to complete complex lattice-type perceptual mazes (10).
Digit Vigilance	Number of target stimuli (the number "6") missed when required to scan two pages of numbers (11).
Recurring Words*	Percentage of words identified correctly as either "new" or "repeated" when words are read using a continuous recognition test format (12).
Grooved Pegboard	Time required to insert 25 grooved pegs into slotted holes (13).
Statin-Insensitive Tests	
Digit Symbol*	Time required to recode numbers into symbols using a key that pairs each of nine digits with a meaningless shape. Time converted to scaled, normalized score (14).
Stroop Interference	Viewing a list of color words printed in an incongruous ink color, participants say each ink color as quickly as possible (seeing "red" printed in blue ink, they respond "blue"). Number correct is converted to a scaled, normalized score (15).
Trail Making B*	Time required to draw a line connecting alternating numbers and letters (e.g., 1-A-2-B) that are arrayed on a piece of paper (16).
Digit Span*	Longest span of digits correctly recalled forwards and longest span recalled backwards. Sum of spans is converted into scaled, normalized score (14).
Complex Figure*	Score on the reproduction of the Rey or Taylor figure, drawn from memory 30 minutes after having copied the figure (17).
Letter Rotation	Number of stimuli (the letters F, L, or R rotated 0°, 30°, 60°, 90°, 120°, 150°, or 180°) misidentified as being either oriented normally or reversed (18).
New Tests	
Mirror Tracing*	Number of errors made when tracing over a star pattern that can be seen only in mirror-reversed view (19).
4-Word Short-Term Memory	Across several trials, the number of words correctly recalled after intervening distraction consisting of serial subtraction arithmetic for 15 or 30 seconds (20).

* Tests on which two alternative forms were used.

ELSEVIER

Effects of nicotinic stimulation on cognitive performance

Paul A Newhouse*, Alexandra Potter and Abhay Singh

Recent advances in studies of nicotinic agents in humans have begun to more carefully define cognitive operations that can be influenced by nicotinic stimulation and/or blockade. Careful separation of the cognitive domains affected by nicotinic stimulation has identified attentional performance as the most likely candidate to be positively influenced by nicotinic receptor activation. Studies of the effects of nicotinic systems and/or nicotinic receptor stimulation in pathological disease states such as Alzheimer's disease, Parkinson's disease, attention deficit/hyperactivity disorder and schizophrenia show the potential for therapeutic utility of nicotinic drugs. In contrast to studies in pathological states, studies of nicotine in normal-non-smokers tend to show deleterious effects. This contradiction can be resolved by consideration of cognitive and biological baseline dependency differences between study populations in terms of the relationship of optimal cognitive performance to nicotinic receptor activity. Although normal individuals are unlikely to show cognitive benefits after nicotinic stimulation except under extreme task conditions, individuals with a variety of disease states can benefit from nicotinic drugs. Attentional function/dysfunction may serve as an endophenotypic therapeutic target for nicotinic drug development.

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Abbreviations

AD	Alzheimer's disease
ADHD	attention deficit/hyperactivity disorder
CNS	central nervous system
fMRI	functional magnetic resonance imaging
MCI	mild cognitive impairment
nAChR	nicotinic receptor
PD	Parkinson's disease
PET	positron emission tomography
VSWM	visuospatial working memory

Introduction

Neuronal nicotinic receptors (nAChRs) are found throughout the central nervous system (CNS). These receptors are composed of two types of subunits, α and β, of which nine α (α2–α10) and three β (β2–β4) have been found in vertebrates [1–3]. Nicotinic innervation of the hippocampus, amygdala and frontal cortex has been demonstrated to be vital to memory function [4•].

Nicotine is one of over 4 000 compounds found in tobacco smoke, among which is found a variety of carcinogens as well as other toxic compounds such as carbon monoxide, heavy metals and cyanide. However, nicotine administered independent of tobacco appears to be relatively safe [5]. There is a large amount of literature showing that nicotine skin patches are safe and have very low abuse liability [6,7].

The behavioral effects of nicotine are complex and belie a simple classification of nicotine as either a stimulant or depressant. Determinants of the effect of nicotine on human behavior include pharmacological variables (e.g. dose, route of administration) and subject differences (e.g. gender, personality variables). Whether, and how, subjects control the administration of nicotine appears to have significant impact on its cognitive effects. Such effects need to be taken into consideration when considering the use of novel nicotinic agonists for possible beneficial behavioral effects.

This review focuses on recent advances in studies of the cognitive effects of nicotine in smokers and normal volunteer populations, and contrasts those studies with trials of nicotinic stimulation in pathological disease states. These disease states represent the most likely targets for nicotinic drug development and include Alzheimer's disease (AD), mild cognitive impairment (MCI), Parkinson's disease (PD), schizophrenia and attention-deficit/hyperactivity disorder (ADHD). In an attempt to resolve the conflicting results of generally negative studies in normal volunteer populations and positive studies in pathological disease states, we focus on an analysis of cognitive and neurobiological baseline differences between study populations. Finally, the concept of attentional function and dysfunction as an endophenotype for nicotinic drug development is introduced, and we offer the hypothesis that this target is orthogonal to disease diagnosis and offers the best target for the therapeutic effects of nicotinic agents.

Nicotinic receptor stimulation as a cognition-enhancing strategy in 'normals'

Studies in humans have spanned several decades and consist mostly of experiments utilizing cigarettes to administer nicotine, usually to smokers deprived of cigarettes for some period of time. Although nicotine might

'improve' performance in deprived smokers, it appears that this improvement is usually limited to restoring pre-deprivation performance, which clearly declines during cigarette withdrawal [8]. Enhancement of normal non-deprived smokers and nonsmokers with nicotine has been more difficult to demonstrate. In studies with humans, nicotine has been shown to improve performance in smokers on attentionally and cognitively demanding vigilance tasks [9–11]. Recent work has shown that attentional improvements are seen even in the absence of nicotine withdrawal effects [12].

Recent studies of the effects of nicotine and/or smoking on cognitive performance in smokers have attempted to mitigate or minimize many of the problems associated with cognitive studies utilizing drug-dependent individuals. Bell, Taylor, Singleton, Henningfield and Heishman [13] confirmed that smoking deprivation impairs cognitive performance and that re-administering cigarettes briskly relieves this performance decrement. Utilizing electrophysiological assessment, nicotine administration to smoking-deprived smokers improved power indices of electroencephalogram arousal, with shortened reaction times increasing P300 (evoked potential occurring 300 msec after stimulus presentation) amplitudes [14]. Under conditions of suboptimal alertness, smokers who were administered nicotine showed improved and constant performance during a sustained choice reaction time task, suggesting that nicotine has an enabling effect on sustained cognitive effort, at least in this population [15]. Utilizing the strategy of administering a transdermal nicotine patch to smokers participating in a smoking withdrawal study, Cook, Gerkovich, Graham, Hoffman and Peterson [16] did not find that nicotine administration mitigated the cognitive effects of smoking cessation and hypothesized that performance decrements noticed by smokers during cessation may be related to affective disturbances rather than cognitive impairment. Utilizing non-deprived smokers, Sakurai and Kanazawa [17] compared smokers with nonsmokers in a variety of memory, calculation and executive function tasks after the smokers had received one or two cigarettes. They found no intergroup differences and suggest that a daily dose of nicotine has little effect on performance. Ernst, Heishman, Spurgeon and London [18] found that smoking history appeared to interact with performance effects in individuals who had been administered nicotine gum, with abstinent smokers performing most poorly and 'never smokers' performing best on a working memory task. Heishman and Henningfield [19] administered nicotine gum across a range of doses to normal nonsmokers both acutely and chronically. Nicotine administration increased the rate of responding and decreased response time, but impaired accuracy on working memory tasks; accuracy also was impaired on visual scanning attention and gross motor coordination.

Functional neuroimaging studies of the effects of nicotine utilizing functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) have suggested that performance enhancement is associated with increased task-induced fronto-parietal-thalamic activation, areas associated with visual attention, arousal and motor activation [20•], or altered lateralization accompanied by reduced cerebral blood flow [21]. A PET study of cerebral glucose metabolism in normal volunteers showed that nicotine administration reduced global glucose metabolism by approximately 10% across most brain regions [22]. Finally, in an intriguing set of studies, Mumenthaler *et al.* [23••] compared the effects of nicotine and the anticholinesterase inhibitor donepezil on the performance of experienced pilots in a flight simulator device. In this highly demanding and stressful cognitive situation, both nicotine and donepezil improved performance substantially with roughly the same effect size, particularly on tasks that required sustained visual attention. Interestingly, a single dose of nicotine improved performance almost as effectively as one month of donepezil administration.

Psychopathological conditions

nAChRs play important roles in the functional impairments of certain neurodegenerative diseases, including AD [24,25]. These associations, although in some cases indirect, are impressive for their longevity and because the associations have been made both by many different investigators and by using more than one approach. The association between AD and nAChRs, although also indirect, suggests that these receptors may mediate important signals that influence the course of the disease. Thus, epidemiological evidence suggests that smokers have a significantly lower incidence of symptoms and diagnosis of AD [26,27]. This inverse relationship even extends to populations with high risk factors for early-onset AD, such as apolipoprotein E gene status [28]. In addition to a strong epidemiological association, many studies have found a decrease in the density of nAChRs in the autopsied brains of AD patients [29–33]. The receptors lost in AD brains are predominantly of the $\alpha 4\beta 2$ subtype, which binds nicotine and other nicotinic agonists with high affinity and is one of the major nAChR subtypes in mammalian brain [34,35]. This decrease in nAChRs is now well-enough established as part of the findings in autopsied AD brains (>10 studies) that efforts are underway in several laboratories to develop neuroimaging ligands for possible use in early diagnosis or for following the course of the disease [36–42].

Newhouse, Potter, Kelton and Corwin [24] recently reviewed the evidence supporting the importance of nAChRs in the development of AD. In summary, a loss of nicotinic binding sites has been demonstrated in AD patients to be linked to the pathological hallmarks of plaques and tangles. The loss of nAChRs appears to be associated with decreased cerebral perfusion in AD.

Mild cognitive impairment

MCI is defined as a subjective and objective decline in cognition and function that does not meet criteria for a diagnosis of dementia [43–45] and that represents a transitional state between the cognition of normal aging and mild dementia [46]. Furthermore, recent evidence indicates that people with MCI are at high risk for subsequently developing dementia [47,48]. Amnestic MCI [46] appears to represent the condition most likely to progress to AD, whereas individuals who have multiple domains or non-memory domains impaired might progress to AD and/or other types of dementias. By utilizing criteria for amnestic MCI, long-term follow-up studies have suggested that these individuals progress to dementia at a rate of approximately 12% per year [46]. Stimulation of CNS nAChRs with nicotine could be a promising strategy to ameliorate symptoms of MCI and/or slow or prevent progression to frank dementia. In a recent study of cognitive performance in patients with MCI, White and Levin [49] studied 10 subjects with MCI in a double-blind, placebo controlled, crossover study consisting of two four-week periods separated by a two-week washout period. Subjects were given nicotine patches to wear for 16 hours a day up to 10 mg per day. Nicotine significantly improved ratings of overall performance on the clinical global impression scale, as well as objective tests of attentional function on the Connors Continuous performance test and on the neuropsychology test battery, compared with placebo. Intriguingly, nicotine treatment improved the decision-making portion of reaction time to a greater extent than it did the simple psychomotor speed. This was accompanied by improvements in error performance on the Connors Continuous performance test task. Such results are encouraging and have prompted the launching of a NIA-funded multicenter trial to test the efficacy of one year of transdermal nicotine therapy in MCI. In many respects, MCI might be the optimal diagnosis for which to test the efficacy of nicotinic therapy. The likelihood of relatively large numbers of preserved nAChRs and the relative preservation of attentional, acquisition, encoding and retrieval mechanisms, taken together with the evidence for substantial neuroprotective effects of nicotinic stimulation, make MCI an ideal diagnosis to test the efficacy of long-term, low-dose nicotinic stimulation. The large number of controlled efficacy smoking cessation trials in healthy and diseased subjects and their accompanying enormous safety database provide confidence that this treatment will be well-tolerated and safe.

Nicotinic treatment of cognitive impairment in Alzheimer's disease

In clinical studies, Newhouse *et al.* [50] first showed evidence of improved cognitive functioning (decreased errors) following intravenous injection of nicotine in AD subjects. Nicotine administration by subcutaneous injection was then shown to improve attention-related task performance in AD [51,52]. Two weeks of nicotine skin patch treatment was found to significantly improve cognitive function in AD patients by Wilson *et al.* [53]. These investigators found that the major effect of nicotine was to reduce error performance on the new learning phase of the repeated acquisition test — the same parameter on the same task (although performed differently) that Newhouse, Potter, Corwin and Lenox [54] found to be specifically impaired after the nicotinic antagonist mecamylamine. A four-week trial of transdermal nicotine in AD was performed by White and Levin [55], who showed significant improvement in attentional performance, as measured by a continuous performance task, with consistent improvements in omission errors and improved consistency of reaction time. Another nicotine patch study did not find any differential improvement in short-term memory with nicotine compared with placebo [56]. However, there were significant practice effects in that study which could have limited sensitivity. In a unique and not previously reported combination, subjects that were treated with the cholinesterase inhibitor tacrine were administered nicotine; they showed decreased auditory-evoked potential latencies and increased visual-evoked potential amplitude, suggesting improved sensory detection, attention and/or processing [57]. In the only example of treatment with a novel nicotinic agonist, Potter *et al.* [58] showed significant dose-related enhancement of learning and memory in AD patients after acute treatment with the nicotinic agent ABT-418.

In addition to direct stimulation of nAChRs, nicotine might provide cascading effects through stimulation of the release of a variety of transmitters involved in cognitive function, including acetylcholine, dopamine, norepinephrine, serotonin and glutamate [59]. Augmentation of the activities of the remaining nAChRs might provide therapeutic benefit. However, the loss of nAChRs in AD could also limit the potential for nicotinic therapy. This might underlie the lack of nicotine effect in AD seen by Snaedal, Johannesson, Jonson and Gylfadottir [56] and the limitations in the extent of improvement in studies of nicotine in AD. Nicotine treatment might be more effective in older adults with less severe cognitive impairment and more nAChRs, such as those individuals with MCI. The documented neuroprotective effects of nicotine [60,61] could help attenuate this decline.

Effects of chronic nicotine

An important consideration in the development of treatments for cognitive impairment is that they do not lose effectiveness with chronic treatment. It is often the case that chronic administration of agonists causes downregulation of their target receptors and the development of tolerance; this is not the case with nicotine. nAChRs actually show an increase in number with chronic nicotine administration [62,63]. This might be due to the fact that nAChRs have a desensitization response that prevents

chronic overstimulation. However, there might also be downstream accommodations in neural systems that receive nicotinic innervation, which would result in tolerance. Therefore, one cannot make a complete argument on the basis of the known mechanisms of action. Much more convincing are the data that show continuing nicotine-induced cognitive improvement with chronic administration. With mild to moderate AD, nicotine skin patch-induced attentional improvement did not diminish over four weeks of administration [55].

Parkinson's disease

Changes in cholinergic systems in the CNS have also been shown to occur in the brains of patients with PD. In particular, a similar loss of cholinergic cells in the basal forebrain nuclei to that occurring in AD has been described in PD [64]. Studies have shown a marked reduction in cortical nAChR binding that parallels the degree of dementia in PD and increasing age [33,65]. There is similarity between the cortical nicotinic binding site loss in PD and AD, as well as similar changes in other cholinergic markers. The loss of presynaptic [66] cortical nAChRs might reflect degeneration of cortical projections from subcortical structures, notably the nucleus basalis, pedunculo-pontine and lateral-dorsal tegmental nuclei. In PD, loss of striatal nicotine binding appears to occur early but is not associated with a loss of α4 subunit immunoreactivity. Accumulating evidence both in rodents and in primates suggests that α6-containing nAChRs are present on nigrostriatal dopaminergic neurons, and that these receptors may be the most vulnerable to nigrostriatal damage, at least in primates. nAChR ligands that activate this receptor might be particularly useful in PD therapeutics [67••]. Newhouse and colleagues proposed that nicotinic augmentation might be a useful therapeutic strategy for both the motor and cognitive symptoms of PD [68,69].

Several studies have shown that smokers have a lower than expected incidence of PD, suggesting a protective effect of nicotine [70,71]. Tobacco use is associated with greater numbers of dopaminergic neurons in the substantia nigra pars compacta [72].

Nicotine was examined as a treatment for PD as early as the 1920s [73], although these patients suffered from a form of secondary parkinsonism caused by encephalitis lethargica. Some patients in the study showed improvement in rigidity but there were also severe side effects, including seizures, after large doses of nicotine. Fagerström, Pomerleau, Giordani and Stelson [74] reported a detailed study of two patients who had nicotine gum and patch added to their PD therapy. Using a single-subject placebo-control reversal design, improvement was associated with nicotine dosing and involved diminished tremor and disorganized thinking in one patient, and lessened bradykinesia and increased energy in the other. Kelton, Kahn, Conrath and Newhouse [75] examined the acute and chronic effects of nicotine in PD patients over several weeks. Positive effects of nicotine appeared in all motor performance tasks including the pronation/supination task, the stand/walk/sit task and the finger dexterity task. There was a statistically significant improvement on the choice reaction time task in the motor component. Particularly interesting was the persistence of some of the effects of nicotine even several weeks after the cessation of drug administration. These results suggest that nicotine administration has positive effects on certain cognitive and motor aspects of PD, particularly in the area of attention and motor speed. However, a randomized, double-blind placebo-control study of transdermal nicotine added to standard antiparkinsonian therapy for three weeks did not find statistically significant improvements in motor performance on clinical rating scores [19]. In contrast, studies of nicotine gum in PD patients have shown positive effects [76,77].

In addition to nicotine, other novel nicotinic agonists have been examined for effects in PD. SIB-1508 and its racemate, SIB-1765F, are subtype-selective nicotinic agonists particularly to α4β2-containing nAChRs [78]. These compounds appear to have greater efficacy than nicotine at releasing dopamine from striatal slices. SIB-1765F potentiated the positive locomotor effects of L-dopa in a reserpine model of PD in rats [79] with a rapid onset of action. The compound produced a small improvement in locomotion when administered alone; however, the effect was much greater when combined with L-dopa. SIB-1508Y, an isomer of SIB-1765F, was more potent in this model and has also shown positive activity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkey model of PD [80]. A dose of SIB-1508Y which was inactive by itself caused significant improvement in cognition and motor aspects of task performance when administered simultaneously with L-dopa.

Schizophrenia

Research in normal smokers and in patients with schizophrenia has indicated that cigarette smoking or nicotine administration may improve cognitive functioning, including memory, attention and spatial perception [81]. Epidemiological studies have shown a higher rate of smoking among schizophrenic patients (90%) compared with the general population (20–30%), and a lower rate of smoking cessation among schizophrenic patients [82]. It has been hypothesized that the high rate of smoking among schizophrenic patients might result, in part, from the ability of nicotine to ameliorate some of the cognitive deficits associated with schizophrenia [83].

Schizophrenic patients exhibit an auditory sensory gating deficit characterized by diminished suppression of P50 auditory-evoked response to repeated stimuli. Nicotine

transiently corrects the diminished gating response of P50 auditory-evoked potential in schizophrenic patients and their non-smoking relatives [84]. Clozapine, but not typical antidopaminergic antipsychotic drugs, improves gating of the P50-evoked response [85], and it has been suggested that modulation of smoking by clozapine treatment may, in part, be caused by similar mechanisms. The fact that these attentional abnormalities also occur in non-psychotic relatives of schizophrenic patients suggests a genetic basis for this deficit. The P50 inhibition deficit in schizophrenia has been linked to chromosome 15q13–14 in the region of the α7 subunit gene [86]. Postmortem studies have shown a reduction in the number of α-bungarotoxin-sensitive (α7-containing) nAChRs in the hippocampal region of schizophrenic patients [87], which appear to be secondary to polymorphisms in the α7 promotor [88•]. Another form of cognitive deficit associated with schizophrenia is latent inhibition, in which pre-exposure to a stimulus inhibits conditioning to that stimulus [89]. It has been suggested that smokers have enhanced latent inhibition, which is dependent upon the pre-exposure parameters [90].

Schizophrenic patients have impairments in other cognitive domains, including deficits in visuospatial working memory (VSWM), which is partly mediated by dopamine in the prefrontal cortex. Smoking abstinence differentially alters VSWM in schizophrenic versus control smokers, and cigarette smoking has beneficial effects on VSWM in schizophrenic, but not control, smokers [91]. Higher doses of nicotine patch improved reaction time, but not accuracy, in a spatial rotation task, and also improved performance on a visual-match-to-sample task in schizophrenic patients treated with haloperidol [92]. Recently, Smith, Singh, Infante, Khandat and Kloos [93•] have shown increased performance on two-choice reaction time, spatial rotation (accuracy/reaction time) and visual-match-to-sample tasks from the automated neuropsychological assessment battery (ANAM) with both high and denicotinized cigarettes in schizophrenic patients. Active nicotine nasal spray improved accuracy on a spatial organization task, and tended to improve some measures of verbal memory (paired words and short story from Randt memory test) and two-choice reaction time in schizophrenic patients. The differences in results between the above mentioned studies might be caused by different forms of smoking and/or to the fact that most of the patients in the Smith study [93•] were on atypical antipsychotic medications.

Attention deficit/hyperactivity disorder

Adults and adolescents who are diagnosed with ADHD smoke at significantly higher rates than comparable people in a community sample, and have lower quit ratios (percentage of ever-smokers who are ex-smokers) than the general population (23% versus 51.6%) [94]. In this study, there was a relationship between current smoking status and retrospective reports of ADHD symptoms, with current smokers recalling a greater number and greater severity of ADHD symptoms in childhood. A prospective study of tobacco smoking and substance dependence [95] found that, by age 17, 46% of adolescents with ADHD were smoking cigarettes daily compared with 24% of age-mate controls. This finding continued into adulthood where 35% of adult subjects with ADHD were smokers as compared with 16% of age-matched controls. These findings raise the possibility that adolescents with ADHD may be using cigarette smoking to relieve some of the symptoms of ADHD.

There is an emerging body of literature examining the therapeutic effects of nicotinic stimulation on the symptoms of ADHD. Levin *et al.* [96] studied the acute effects of transdermal nicotine and placebo in adults with ADHD (both smokers and non-smokers). They reported significant improvements in self-rated vigor and concentration and observer-rated illness severity for both subject groups (i.e. smokers and non-smokers). In addition, they found improvements in speed of responding for both the smokers and non-smokers, and a reduction in variability of reaction time for the smokers. In a second study, Levin, Conners, Siliva, Canu and March [97] studied the effects of chronic (four week) nicotine administration compared with methylphenidate treatment, placebo treatment and a combination of nicotine and methylphenidate in 40 adults with ADHD. They found nicotine to significantly reduce clinician ratings of severity of symptoms, and to decrease self-reported symptoms of depression. They found sustained improvement in the variability of reaction times on a continuous performance task. Wilens *et al.* [98] studied a novel cholinergic channel activator (nicotinic agonist), ABT-418, in 32 adults with ADHD for treatment of their symptoms. This study employed a crossover design with each subject receiving two double-blind three-week treatment periods with placebo and ABT-418. Significant improvements in subjective ratings of attentiveness and observer-rated illness severity on a clinical global impressions scale were seen following treatment with ABT-418.

The cognitive deficits in attention in ADHD are not in the areas of information processing or in perceiving information, but are seen in motor inhibition, motor control and in anticipating events [99,100]. Current views of ADHD hold failures of cognitive/behavioral inhibition as the central deficit of this disorder [101]. Potter and Newhouse [102] have recently examined changes in behavioral inhibition following nicotine administration to eight non-smoking adolescents with ADHD. In this study, subjects were administered acute transdermal nicotine (7 mg for 60 min), oral methylphenidate or placebo on three separate study days. Nicotine was associated with significant improvements in stop signal reaction time (a measure of the speed of inhibition). This

effect was comparable to the size of change seen after a single dose of oral methylphenidate and was not associated with general improvements in performance on this task, as overall speed and accuracy were not different from placebo. This study also found improved cognitive/behavioral inhibition associated with nicotine administration on the Stroop task, with a smaller Stroop effect (the cost of inhibiting) following nicotine, but not methylphenidate or placebo, treatment. In addition, nicotine was associated with decreased subject ratings of irritability and observer ratings of anxiety.

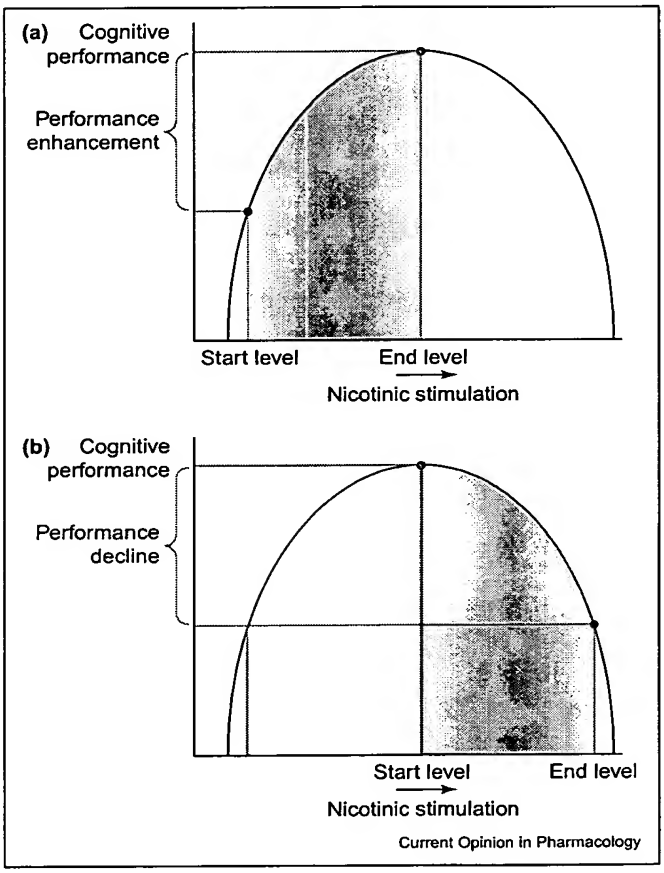
These initial studies indicate that nicotinic treatment may be useful for the symptoms of ADHD. The well-known effects of nicotine on attention might include positive effects on cognitive/behavioral inhibition, which is the core cognitive deficit of ADHD. Current standard pharmacological treatment for ADHD consists mainly of psychostimulants (e.g. methylphenidate), which are presumed effective via their effects on dopamine. Nicotine has been shown to increase the release of several neurotransmitters, including dopamine [103]. nAChRs might serve to regulate dopamine release in both striatal and mesocortical pathways [104,105]. Levin, McGurk, Rose and Butcher [106] have performed an extensive series of studies suggesting complex interactions with several possible anatomical loci for the site(s) of interaction, including both limbic and hippocampal areas, as well as descending projections to dopamine-containing areas of the mesencephalon via the medial habenula. Thus, is it possible that nicotine is exerting positive effects on inhibition and sustained attention in ADHD by enhancing the dopaminergic tone of both the striatal-frontal and mesocortical dopaminergic systems. In support of this conclusion, Solanto [107] in a recent review concluded that the majority of cognitive symptoms of ADHD (including behavioral inhibition) is mediated by the prefrontal cortex, and that stimulant medication might affect cognition by acting at D1 and D2 receptor sites to optimize the neurochemical environment.

Does nicotinic stimulation enhance cognitive functioning or performance?

A review of the recently published works on the effects of nicotine on cognitive function and performance in humans could lead to some confusion because of the contradictory conclusions of these studies. There seems to be an almost equal number of studies that show either performance enhancement or impairment by nicotine. A careful look at the nature of these disparate studies reveals clues to understanding the seemingly contradictory nature of research in this area.

Studies that tend to show impairment generally use normal non- or never-smokers as subjects, even studies with chronic use. These studies tend to conclude that nicotine does not improve cognitive functioning and most often impairs it. In contrast, studies that tend to show improvement generally utilize smokers or clinical populations of subjects. These studies generally demonstrate and/or conclude that nicotine has cognitive-enhancing effects. These disparate results can be resolved by considering that the findings reflect the differing populations utilized for the experiments. These populations can be expected to show quite different responses to nicotine from the principles of rate dependency or baseline effects [108] of nicotine (e.g. the Yerkes-Dodson principal). This is illustrated in Figure 1. Cognitive performance can be

Figure 1



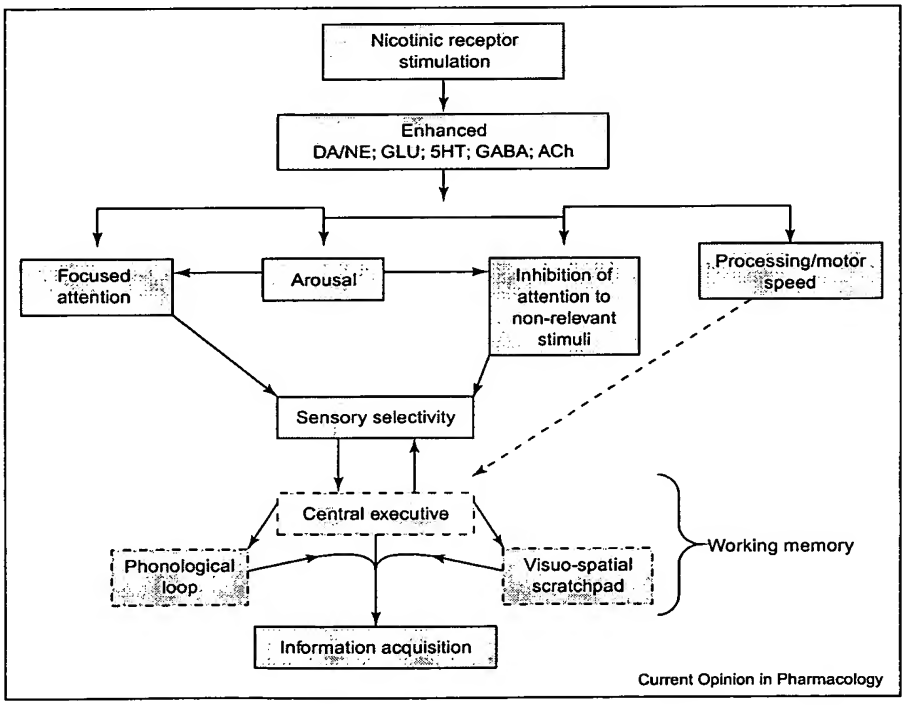
This figure illustrates two situations in which an equivalent degree of nicotinic stimulation produces opposite effects and illustrates the general principle that the results of nicotinic stimulation are a reflection of baseline performance level. The results of nicotinic stimulation, like many biological systems, can either increase or impair function (Yerkes-Dodson principal). **(a)** Illustrates an example of an individual whose cognitive performance is impaired or for whom task demands do not match the level of ongoing nicotinic stimulation. The nicotine administration or nicotinic receptor stimulation produces an improvement in cognitive performance, bringing performance to near optimal levels. **(b)** Illustrates a different scenario where the individual performance is already at or near optimum levels. In this case, the same degree of nicotinic stimulation as in **(a)** produces impairment of performance. This illustration might help to explain apparently paradoxical results of cognitive studies in differing populations.

envisioned as a curvilinear function related to nicotinic stimulation, with intermediate levels of stimulation producing optimal performance and either low or high levels of stimulation impairing performance. If an individual subject is performing suboptimally because of a disease state or impairment (e.g. AD), his performance will be enhanced by increased nicotinic stimulation (Figure 1a). However, if an individual subject is already performing at or near their optimal level of performance, increasing nicotinic stimulation will produce deterioration in cognitive functioning (Figure 1b). The same analysis can apply if the individual is normal but the task demands are severe. If the task is demanding enough in terms of attention, especially over a period of time, then the individual might move back to the left in terms of the performance curve, and optimal performance will require enhanced nicotinic stimulation.

Studies of normal volunteers, especially non- or never-smokers, are unlikely to show cognitive improvement with nicotinic stimulation because of the fact that these individuals are likely to be operating at or near their optimal level of performance, particularly in the setting of experimental paradigms with, for example, pre-training for cognitive tasks and financial rewards for participation. In addition, this might reflect differences between non-smokers and smokers in the underlying neurobiology and/or efficiency of the nicotinic system, and suggests that some of the reasons why subjects smoke may be, in part, related to the degree of improvement in cognitive performance that is seen with nicotinic stimulation.

The preponderance of evidence suggests that stimulation of nAChRs is most easily detected by effects on attentional systems and, to some extent, psychomotor speed. The most well-documented effect of nicotine is intensifying or sustaining attention to stimuli or tasks over a prolonged period of time. In addition, there is evidence from studies of individuals with disorders such as schizophrenia and ADHD that nicotinic stimulation enhances selective attention, sensory detection and inhibitional processes in attention. Positive effects of nicotine on learning or memory might be mediated by its effects on attentional functioning (Figure 2). Learning and memory require acquisition, encoding, storage and retrieval; however, attention is the 'front end' of this process, and adequate attentional functioning is a primary requirement.

Figure 2



Proposed model for nicotinic receptor stimulation effects on neurotransmitter function and attentional function. In this model, nAChR stimulation is presumed to lead to enhanced neurotransmitter release in particular brain areas relevant to arousal, sustained attention, inhibitional processes and processing/motor speed. Sensory selectivity is conceptualized as being secondary to improved attentional performance. These processes are thought to impinge on the 'central executive' component of Baddeley's model [110] for working memory leading to improved acquisition of information.

Conclusion: attention as a nicotinic endophenotype

In summarizing research done on nicotine and cognition, Warburton and Rusted [109] concluded that the most robust effects of nicotine are seen in tasks that have a high attentional requirement (i.e. memory enhancement might be a consequence of improved attentional functioning). Attention and related processes can be thought of as an endophenotype for nicotinic stimulation and consequently drug development. Attention, central processing impairment and executive dysfunction might be orthogonal to the underlying neuropsychiatric diagnosis and should be considered as an independent target for nicotinic drug development across diagnostic categories. Particular attentional deficits in different diagnoses could still respond to nicotinic stimulation; however, the parameters for assessing improvement might be quite different between disease states and will require careful attention to receptor subtype-specific agents, dosing regimens and outcome measures in clinical trials. Paying careful attention to the issue of baseline-dependency in treatment response will be vital to ensure appropriate interpretation of experimental results, both for studies of normal individuals and for those with disease states. Targeting specific populations that are already impaired is much more likely to reveal potential benefits of nicotinic stimulation. Studies of normal or unimpaired individuals with nicotinic drugs are unlikely to show cognitive benefit except under extreme task demands.

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